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**14. ABSTRACT**

Major advances have been made in two main areas. Firstly, Receptor tyrosine kinase-like orphan receptor-1 (ROR1) was identified as a tumor antigen expressed on ovarian cancer (OvCa), but not expressed on normal tissue(s). Second generation chimeric antigen receptors (CARs) were designed with CD3z and either CD28 or CD137 endodomains fused to the antigen-binding region of a ROR1-specific monoclonal antibody (clone 4A5). CARs were stably expressed in T cells following *Sleeping Beauty* SB11 transposition and propagation on ROR1$^+$ artificial antigen presenting cells (aAPC). Re-directed cytolysis of ROR1$^+$ OvCa cell lines by CAR$^+$ T cells was demonstrated. Secondly, the anti-tumor activity of $\gamma\delta$ T cells was harnessed to kill OvCa. Our aAPC were used to massively expand for the first time a polyclonal population of $\gamma\delta$ T cells for immunotherapy. OvCa cell lines and xenografts were eliminated by these massively expanded polyclonal $\gamma\delta$ T cells. $\gamma\delta$ T cells are HLA-unrestricted and rapid expansion with our novel aAPCs may also have utility as off the shelf therapy for Ebola virus and other virus and bacteria mediated outbreaks. Based on this work, a ROR1 CAR clinical trial has been registered at ClinicalTrials.gov and opened in January 2015 (NCT02194374).

**15. SUBJECT TERMS**

ROR1, $\gamma\delta$ T cells, adoptive T cell therapy, ovarian cancer, chimeric antigen receptor (CAR), Sleeping Beauty

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TABLE OF CONTENTS

## INTRODUCTION

**Ovarian cancer, hypoxia, and tumor antigens.** Relapsed ovarian cancer (OvCa) is refractory to current therapies in many cases and remains a lethal disease for women worldwide.[1, 2] Advanced ovarian tumors have significant areas that are hypoxic (low $O_2$ concentration), which can lead to therapeutic escape and relapse in women who initially respond to therapy.[3-5] Therapies targeting tumor-associated antigens (TAAs) expressed in both hypoxic and normoxic regions of ovarian tumors could dramatically increase survivorship in women with advanced disease. c-Met, hepatocyte growth factor receptor (HGFR), has been identified as a candidate TAA because of its well-described expression on OvCa that increases in hypoxia.[6-8] However, c-Met is also expressed on normal tissues, such as liver and lung,[9] thereby complicating its utility as a TAA for cell-based therapy. In contrast to c-Met, the TAA receptor tyrosine kinase-like orphan receptor-1 (ROR1) is not expressed on normal adult tissues, but is expressed on a variety of cancers, including OvCa.[10-12] ROR1 expression was maintained in hypoxia in OvCa cell lines (**Figure 1**), suggesting that targeting ROR1 may be a safe and effective strategy for OvCa immunotherapy. Based on this work, a ROR1 CAR clinical trial has been registered at ClinicalTrials.gov and opened in January 2015 (NCT02194374).

**Chimeric Antigen Receptors.** Chimeric antigen receptors (CARs) are employed to re-direct T-cell specificity to TAAs. They are fashioned by fusing T-cell signaling endodomains (CD3ζ, CD28, and CD137) to a single chain variable fragment (scFv) derived from a monoclonal antibody (mAb) with specificity to a cell surface TAA. Upon docking with TAA the introduced CAR activates genetically modified T cells outside of the constraints of major histocompatibility complex (MHC) interaction with the endogenous T-cell receptor (TCR). The CAR contains both signal 1 (CD3ζ) and signal 2 (CD28 or CD137) to achieve full T-cell activation. Clinical trials are investigating the therapeutic efficacy of CAR-based therapies.[13] Patients with refractory B-cell malignancies who were recently treated with CD19-specific CAR+ T cells employing a CD137 endodomain achieved complete responses.[14, 15] Thus, ROR1 was targeted in this study with CAR+ T cells expressing either CD28 (ROR1RCD28) or CD137 (ROR1RCD137) endodomains (**Appendix 1**).

**Adoptive T-cell therapy, graft-versus-host disease, and γδ T cells.** Allogeneic adoptive T-cell therapy (allo-ACT) is a cancer treatment modality used in clinical trials. Allo-ACT is particularly useful when autologous T cells cannot be generated, *e.g.*, after/during chemotherapy.[16, 17] However, there is the risk of graft-versus-host disease (GvHD) in allo-ACT, where donor TCRαβ inappropriately recognizes the host MHC leading the infused T cells to attack normal tissues. It is difficult to predict GvHD incidence because specificity of the donor-derived TCRαβ chains are uniquely educated within individuals.[18] T cells can be dichotomized into two main subsets based on their TCRs. The conventional αβ T cells express TCRαβ and constitute ~95% of the circulating T-cell pool. In contrast, γδ T cells are less frequent (1-10% of peripheral-blood-derived T cells) and are characterized by TCRγδ. While αβ T cells recognize peptides in the context of MHC, γδ T cells do not necessarily recognize MHC and most of the antigens they recognize are outside of MHC presentation altogether. Therefore, γδ T cells have a reduced risk for GvHD and appear to be a safer option for allogeneic allo-ACT compared with T cells expressing αβTCR.[19]

**γδ T-cell numeric expansion and utility in cancer therapy.** Bisphosphonates have been used to propagate mono- and oligo-clonal subsets of human peripheral blood-derived γδ T cells. The utility of clinical-grade bisphosphonates in γδ T-cell expansion was serendipitously discovered when patients with bone diseases, e.g. osteoporosis, sarcoma, etc…, were treated with bisphosphonates to regenerate their bone and sizeable expansions of γδ T cells were subsequently observed *in vivo*.[20] The bisphosphonate-derived Vδ2 T cells are reactive to metabolites in the cholesterol synthesis pathway and display natural anti-tumor reactivity, because tumors are commonly dependent upon cholesterol-rich lipid rafts for growth factor signaling. Moreover, γδ T cells are regarded in general as a critical part of the natural anti-tumor response.[21, 22] Clinical trials have investigated the efficacy of bisphosphonates in tumor therapy with some promising results in both hematological and solid cancers, including OvCa, but were not curative.[23] In contrast, long term survivorship of patients following allogeneic TCRαβ-depleted hematopoietic stem-cell transplantation (HSCT) was predictive based on increased frequency of peripheral γδ T cells that were primarily of Vδ1 origin.[24-26] Polyclonal γδ T cell expansion has not been achieved, which limits the ability to use both anti-tumor subsets of γδ T cells in anti-cancer therapy. A major problem with attempting to *ex vivo* propagate γδ T cells to clinically-appealing numbers is that conventional strategies to expand αβ T cells, *e.g.*, with high-dose IL-2 and OKT3 (anti-CD3), do not support long-term growth of γδ T cells in tissue culture. Furthermore, there are very limited starting

quantities of γδ T cells (1-10% of peripheral T cells). The expansion schema used in our lab centers around using K562-derived artificial antigen presenting cells (aAPC) that express cytokines, co-stimulatory molecules, and TAA as feeder cells to support T-cell growth to clinically relevant numbers. K562 cells are natural targets for allogeneic γδ T cells, and for the first time, our K562 genetically modified aAPC enables massive expansion of all three subsets of γδ T cells, with similar kinetics as αβ T cells. Our γδ T cells can now be massively expanded on our aAPC in compliance with current good laboratory practice (cGLP) and current good manufacturing practices (cGMP) and thus can be rapidly translated into clinical trials. The first clinical trials using *Sleeping Beauty* transposition and aAPC technology is currently open at MD Anderson for treating B cell leukemia with allogeneic αβ T cells re-directed to leukemia with a CD19-specific CAR.

***Sleeping Beauty* transposition and aAPC-based T-cell expansion.** As with the CD19-specific CAR, ROR1 CARs can be expressed in T cells with the *Sleeping Beauty* transposase/transposon gene transfer system. *Sleeping Beauty* transposition is a non-viral gene cut-and-paste mechanism where the transposase enzyme is transiently expressed in the cell, makes excisions at inverted repeats flanking the transposon containing the CAR, and integrates the excised transposon into TA repeats within the genome.[27] After a few days, the transposase enzyme is no longer expressed, and long-term stability and expression of the transposon can be readily achieved. Nucleofection is used to electroporate peripheral blood mononuclear cells (PBMC) with CAR transposon and *Sleeping Beauty* SB11 transposase, and stimulations with γ-irradiated aAPC are applied weekly to the cells along with recombinant interleukin-2 (IL-2) and interleukin-21 (IL-21).[28] The aAPC culture imposes selective pressure for selective CAR$^+$ T-cell propagation through (i) corresponding antigen expression, (ii) co-stimulation, and (iii) cytokine support, and after four weeks the culture contains >90% CAR$^+$ T cells at clinically relevant numbers (**Appendix 2**). The electroporated/propagated CAR$^+$ T cells kill tumors and secrete pro-inflammatory cytokines in a CAR- and antigen-restricted manner, and can allow for conditional production of pro-inflammatory molecules in a canonically immunosuppressive tumor microenvironment.

**IL-17 and ovarian cancer.** IL-17-polarized T cells exhibit anti-tumor efficacy and are sought after cell lineage for adoptive T cell therapy. More specifically, IL-17 expression and presence of IL-17 producing cell types in tumors are positively associated with survivorship in ovarian cancer.[29, 30] IL-17 is well described to induce pro-inflammatory processes, which can be advantageous in cancer therapy.[31] CD4$^+$ T cells, CD8$^+$ T cells, and γδ T cells have been described as IL-17 producers, and are named Th17, Tc17, and Tγδ17, respectively.[32] Importantly, Th17 cells are inversely correlated with regulatory T cells (Tregs), and Tregs often promote tumor growth.[33, 34] It was recently shown that inducible co-stimulator (ICOS) polarized CD4$^+$ T cells towards Th17 lineage, and that CD28 co-stimulation reversed the polarization and made the CD4$^+$ T cells express interferon-γ (IFNγ).[35] This is important for the current aAPC expansion strategy because the aAPC express CD86, a ligand for CD28, and most aAPC-expanded T cells are restricted to IFNγ production and not IL-17. γδ T cells are also known to polarize towards either IFNγ or IL-17 producing lineages, although the co-stimulatory requirements are unknown. CD27 expression predicts for IFNγ producing γδ T cells, whereas CD27$^{neg}$ γδ T cells are commonly IL-17 producing lineages.[36] Optimization of the co-stimulatory molecules on the aAPC could allow skewing towards ideal cytokine production and anti-tumor effect that can be induced in the hypoxic environment by T cells.

**Summary.** This project accomplished (i) targeting of ovarian cancer with ROR1-specific T cells under conditions of normoxia and hypoxia and (ii) optimization of the co-stimulation requirements for maximum anti-tumor efficacy and pro-inflammatory cytokine production by the T cells, especially IL-17, in the ovarian tumors. Our *Sleeping Beauty* gene transfer and aAPC technology were integral in evaluating the hypotheses, and data generated by this study will serve as the basis for initiating a ROR1 CAR T-cells clinical trial for the safe and effective treatment of disease-resistant ovarian cancer. Furthermore, for human γδ T cells, we (iii) massively expanded three γδ T cells subsets in culture with our aAPC, and (iv) showed our expanded human γδ T cells killed OvCa tumor cells in vitro. T-cells expanded on aAPC are in clinical trials so we have a direct path to clinical translation of our γδ T cells for ovarian cancer. Based on this work, a ROR1 CAR clinical trial has been registered at ClinicalTrials.gov and opened in January 2015 (NCT02194374).

**KEYWORDS**

ROR1, γδ T cells, adoptive T cell therapy, ovarian cancer (OvCa), chimeric antigen receptor (CAR), artificial antigen presenting cells (aAPC), Clinical trial NCT02194374

# BODY

We **hypothesized** that cell-based therapies for OvCa can be generated by (i) targeting ROR1 with CAR^{+} T cells and (ii) expanding polyclonal $\gamma\delta$ T cells for clinical use where optimization of their endogenous co-stimulation would maximize anti-tumor efficacy.

Specific Aim #1: To evaluate the ability of ROR1-specific CAR to re-direct the specificity of T cells to kill OvCa. Recent clinical trials treating refractory B-cell malignances with CD19-specific CAR^{+} T cells achieved complete responses using a CD137 endodomain instead of chimeric CD28.[15] It is unknown whether the same would apply to targeting ROR1. Two second generation CAR transposons were generated with CD28 (ROR1RCD28) and CD137 (ROR1RCD137) endodomains[14, 15]. The following ovarian cancer cell lines were kindly given to us by Dr. Bast (UTMDACC): A2780, EFO21, EFO27, IGROV1, OC314, and UPN251. Identities of all cell lines were confirmed by STR Fingerprinting (Characterized Cell Line Core, UTMDACC) and were found to be mycoplasma free. Flow cytometry was used to analyze ROR1 antigen levels on the available OvCa cell lines following three days growth in normoxia (20% $O_2$) or hypoxia (1% $O_2$). As anticipated, ROR1 was expressed on 5 of 6 cell lines tested and expression was maintained or increased in hypoxic conditions (Figure 1). This was the first report of ROR1 expression in hypoxia to our knowledge, and corroborated previous reports of ROR1 expression in OvCa.[12] After establishing that ROR1 is expressed in both high and low oxygen tension, OvCa was targeted with ROR1-specific CAR^{+} T cells. Healthy donor-derived peripheral blood mononuclear cells (PBMC) were electroporated with *Sleeping Beauty* transposase and either ROR1RCD28 or ROR1RCD137 transposons (Appendix 1) then stimulated with ROR1^{+} aAPC, IL-2, and IL-21 (Appendix 2). Sham electroporations were be sorted and stimulated in parallel with OKT3 (agonistic CD3 antibody)-loaded aAPC to propagate CAR^{neg} T cells for negative controls. aAPC were phenotyped for antigens (CD19 and ROR1), membrane bound IL15 (fused to IL-15R$\alpha$), co-stimulatory molecules (CD86 and CD137L), and Fc receptors (CD32 and CD64) before stimulations (Appendix 3). After 36 days of expansion on aAPC, >$10^9$ CAR^{+} T cells were propagated from $10^6$ CAR^{+} T cells at the start of the culture (Figure 2). Stable CAR expression was observed with both ROR1RCD28 and ROR1RCD137 indicating that there was imposed selective pressure for CAR expression though cognate antigen expression on aAPC (Figure 3). Extended phenotypic analysis for CD3, CD4, CD8, CD56, CD27, CD28, CD62L, CCR7, CD38, CD95, TCR$\gamma\delta$, and TCR$\alpha\beta$ were evaluated by flow cytometry. Interferon-$\gamma$ (IFN$\gamma$) expression were evaluated by intracellular cytokine staining following TCR stimulation (PMA/Ionomycin) and CAR stimulation from co-culture with EL4-ROR1^{neg} and EL4-ROR1^{+} tumor targets. Ovarian cancer cell lines (A2780, EFO21, EFO27, IGROV1, OC314, and UPN251) were profiled for ROR1 expression in normoxia (20% $O_2$) and hypoxia (1% $O_2$). Four-hour CRA was used to evaluate cytotoxicity against the OvCa and EL4 tumor targets in normoxia and hypoxia. OC314 (ROR1^{+}) cell line was genetically modified to express mKate red fluorescent protein and *firefly Luciferase* (*ffLuc*) to measure tumor burden by fluorescence and bioluminescent imaging (BLI), respectively. *In vivo* tumor clearance of established OC314-mKate-*ffLuc* xenografts (intraperitoneal, i.p.) by CAR^{+} T cells (i.p.) in immunocompromised (NOD.*scid.*$\gamma$c$^{-/-}$; NSG) mice were monitored by non-invasive BLI following subcutaneous (s.c.) D-Luciferin administration. Immunohistochemistry and fluorescence were used to corroborate BLI data post-mortem. Both ROR1RCD28 and ROR1RCD137 displayed minimal killing of EL4-ROR1^{neg} cells (**Figure 4A**) but significantly higher killing of EL4-ROR1^{+} cells compared to CAR^{neg} T cells (**Figure 4B**). Similarly, A2780 (ROR1^{neg}) OvCa cells were lysed at the same levels by CAR^{+} T cells as CAR^{neg} T cells (**Figure 4C**), whereas EFO27 (ROR1^{+}) OvCa cells were significantly killed by CAR^{+} cells compared to CAR^{neg} T cells (**Figure 4D**). Thus, CAR^{+} T cells were specifically re-directed to ROR1 expressed on OvCa. As ROR1 expression on OvCa has only recently been elucidated and has not yet been tested as a target for OvCa, ROR1-specific CAR T cells can thus be used for the first time in OvCa treatment.

Specific Aim #2: To assess the inherent cytolysis of $\gamma\delta$ T cells against OvCa. We established that $\gamma\delta$ T cells propagated on aAPC in parallel with CD19-specific CAR[+] $\gamma\delta$ T cells and maintained a polyclonal distribution of $\gamma$ and $\delta$ TCR chains.[37] This was a major advance as only one subset (V$\gamma$9V$\delta$2) of $\gamma\delta$ T cells had been previously expanded for human application, yet other $\gamma\delta$ T-cell subsets exhibit anti-tumor immunity. Thus, studies were initiated to evaluate whether aAPC would drive $\gamma\delta$ T-cell proliferation in the absence of CAR^{+} T cells and if these $\gamma\delta$ T cell would express polyclonal TCR$\gamma\delta$ repertoire.

Healthy donor-derived PBMC were first depleted of NK cells with CD56 microbeads on paramagnetic columns, and then paramagnetically sorted for $\gamma\delta$ T cells with TCR$\gamma$/$\delta$–$\iota$solation kit then stimulated weekly with aAPC

feeder cells and exogenous IL-2 and IL-21. The other fraction of the paramagnetic bead sorting, comprising mainly of αβ T cells, were stimulated with OKT3-loaded aAPC to propagate αβ T cells for negative staining controls. Extended phenotypic analysis of cell surface markers (as in Aim#1 with TCRδ1, TCRδ2, and TCRγ9) was performed at the end of the culture period by flow cytometry to assess TCR distribution and T cell, homing, and memory phenotype in the culture. TCRγδ allele expression was evaluated by our direct TCR expression array (DTEA[46]) using digital probes to quantify mRNA expressing TCR chains and validated by flow cytometry. Cytokine release following TCR stimulation (PMA/Ionomycin) and co-culture with OvCa cell lines was assessed by Luminex multiplex cytokine release assay. Standard 4-hour CRA was used to evaluate cytotoxicity towards OvCa cell lines detailed in Aim#1 with healthy donor-derived B cells serving as negative controls. CAOV3 cell line was genetically modified to express mKate and *firefly Luciferase* (*ffLuc*) as done for OC314 in Aim#1. The ability of i.p. injected γδ T cells to eliminate i.p. established CAOV3-mKate-*ffLuc* xenografts in NSG mice was evaluated – using *non-DoD* funding -- by BLI during the course of the experiment then immunohistochemistry and fluorescence were used to corroborate BLI data post-mortem.

γδ T cells were in limited quantities in the starting PBMC (3.2% ± 1.2%; mean ± SD; n=4), but after sorting and expansion for 22 days on aAPC the cultures were highly pure for γδ T cells (97.9% ± 0.6%) as assessed by co-expression of $CD3^+$ and $TCR\gamma\delta^+$ (**Figure 5A**). Cultures yielded $>10^9$ γδ T cells from $<10^6$ total cells in three weeks of co-culture (**Figure 5B**), which represented $4.9x10^3 ± 1.7x10^3$ fold change over the culture period (**Figure 5C**). Similar results were seen with γδ T cells sorted from umbilical cord blood (UCB) by fluorescence activated cell sorting (FACS) and stimulated as was done with PBMC (data not shown). Altogether this suggested the aAPC, not CAR, led to growth of γδ T cells in the culture system.

Only three TCRγδ chain-specific antibodies are commercially available limiting the detection of γδ T cell repertoire outside of Vδ1, Vδ2, and Vγ9. Thus, the direct T cell expression array "DTEA[46]" was utilized to detect TCR mRNA in the γδ T-cell cultures and flow cytometry was used to corroborate mRNA data, when applicable. All three Vδ alleles, i.e. Vδ1, Vδ2, and Vδ3, were detected by both flow cytometry (**Figure 6A**) and DTEA (**Figure 6C**) with similar frequencies following the trend of Vδ1>Vδ3>Vδ2. The Vδ3 subset was shown to be present within the $V\delta1^{neg}V\delta2^{neg}$ population with DTEA analysis which allowed for inferential detection by flow cytometry.[37] Most Vδ2 cells paired with Vγ9 (**Figure 6B**), as expected, and DTEA revealed that Vγ2, Vγ5, Vγ7, Vγ8 (two alleles), Vγ10, and Vγ11 mRNA were expressed along with Vγ9 in the aAPC-expanded γδ T cell cultures (**Figure 6D**). All work was performed under GLP for direct clinical translation. Thus, we report the first clinically-relevant expansion of polyclonal γδ T cells for human use.

To determine whether γδ T cells would foster an inflammatory environment during therapy, a multiplex analysis (27-Plex Luminex) of cytokines and chemokines was performed on T cells following culture on aAPC. Phorbol myristate acetate (PMA) and Ionomycin mimic TCR activation by stimulating protein kinase C (PKC) and increasing intracellular $Ca^{2+}$ to activate phospholipase C (PLC), respectively.[38, 39] There was no significant production of anti-inflammatory Th2 cytokines IL-4, IL-5, and IL-13, and there was only a small increase in IL-10 production from baseline (**Figure 7A**). In contrast, IL-1Ra, IL-6, and IL-17 were significantly secreted by γδ T cells and have roles together for IL-17 inflammatory responses important γδ T cells in killing OvCa (**Figure 7B**).[31, 40] Moreover, pro-inflammatory Th1 cytokines IL-2, IL-12 (p70), interferon-γ (IFNγ), and tumor necrosis factor-α (TNFα) were all significantly produced by γδ T cells when TCR was stimulated compared to mock stimulated controls (**Figure 7C**). Extremely high expression of chemokines CCL3 (macrophage inflammatory protein-1α; MIP1α), CCL4 (MIP1β), and CCL5 (regulated and normal T cell expressed and secreted; RANTES) were also detected (**Figure 7D**). In aggregate, TCR stimulation in γδ T cells led to a largely pro-inflammatory response desired for cell-based OvCa therapy.

It is of interest to employ γδ T cells for adoptive T cell therapy because they have less risk for graft-versus-host disease (GvHD). γδ T cells did not proliferate or produce IFNγ in response to healthy donor B cells (selected for their relative abundance in PBMC and ability to function as APC) nor did they have any significant killing of normal cells (**Figure 8; upper left**). Proliferation, IFNγ production, and killing were observed in positive controls, which attests to their functional capacity while leaving healthy cells untouched (data not shown). In addition to having fewer responses to normal cells, γδ T cells displayed an inherent ability to kill ovarian cancer. All OvCa cell lines tested in standard 4-hour chromium release assays were lysed by γδ T cells but healthy B cells were not killed (**Figure 8**). The order of killing tumor cell lines was cell line and donor dependent, which could not be directly correlated to a particular Vδ subset of deference to a certain

combination of Vδ lineages (**Figure 8**). In aggregate, γδ T cells showed broad anti-tumor effects with limited reactivity to healthy cells.

Lastly, the ability of γδ T cells to target and eliminate established OvCa xenografts *in vivo* was evaluated, using *non-DoD* funding. NSG mice were used for their ability to accept human tumor xenografts well and were injected with CAOV3-*ffLuc*-mKate tumor cells i.p. then randomized into treatment groups. Stable disease was established after 8 days of engraftment and either PBS (negative control) or γδ T cells (escalating doses) were administered i.p. to the mice. Tumor burden was monitored during the experiment with non-invasive bioluminescence imaging (BLI) following D-luciferin administration (**Figure 9A**). γδ T cells significantly (p = 0.0005) eliminated established CAOV3 tumors during the course of the experiment whereas mice treated with vehicle (PBS) retained high tumor burden (**Figure 9B-C**). Persistent OvCa disease was evident in the PBS group by (i) flux maintenance to day 43 (**Figure 9B**), (ii) increase in flux to day 79 (**Figure 9D**), and (iii) death of mice (n=3). In contrast, mice treated with γδ T cells displayed significantly lower CAOV3-*ffLuc*-mKate flux after 79 days post-engraftment compared to the day prior to treatment (day 7 post-engraftment) and no mice died in this group (**Figure 9D**). Thus, polyclonal γδ T cells were effective in treating OvCa *in vivo* and represent an attractive approach to cell-based OvCa treatment.

Specific Aim #3: Examine the role of endogenous co-stimulation for pro-inflammatory γδ T cell expansion in both normoxia and hypoxia. In order to maximize the anti-tumor efficacy of γδ T cells for OvCa therapy, it is important to distinguish what molecules on aAPC drive their proliferation. The aAPC discussed in Aims #1 and #2 co-expressed CD86 and 4-1BBL co-stimulatory molecules, which were high value targets for these studies. It was also advantageous to examine IL-17/IFNγ polarization and growth in hypoxia in the same studies. Reports in αβ T cells show that CD70 and CD86 led to IFNγ production but ICOS-L correlated with IL-17. Whether the same is true for γδ T cells is not currently known nor is their ability to proliferate in hypoxia with aAPC as a stimulus.

Normal donor PBMC were sorted for γδ T cells (TCRγ/δ isolation kit) and expanded in parallel in 1% $O_2$ and 20% $O_2$ on aAPC for 9 days with aAPC expressing differing co-stimulatory ligands: (i) none, (ii) CD70, (iii) CD86, (iv) CD137L (4-1BBL), or (v) CD275 (ICOSL). Co-cultures were either given no cytokine, IL-2, IL-21, or both IL-2 and IL-21. After stimulation, cells were counted for proliferation using trypan blue exclusion. Gene expression was profiled using the NanoString nCounter platform to identify candidate genes crucial for expansion in hypoxia. Polarization towards IL-17 or IFNγ producing lineages was evaluated following expansion by flow cytometry and intracellular cytokine staining. Cytolytic potency of cells expanded on different co-stimulatory ligands were evaluated by [51]Chromium release assay (CRA) when targeted to ovarian cancer cell lines in both hypoxia and normoxia. Expanded cells were either mock activated or stimulated with leukocyte activation cocktail (LAC; PMA/Ionomycin) in the presence of secretory pathway inhibitor GolgiPlug for 6 hours at 37°C in normoxia or hypoxia then stained for CD3, TCRγδ, IFNγ, and IL-17 and analyzed by flow cytometry. As anticipated, the γδ T cells did not appreciably expand in the absence of cytokine or aAPC. However, the addition of both IL-21 and IL-2/IL-21 led to γδ T-cell numeric expansion especially when aAPC expressed 4-1BBL and with a combination of CD86 and 4-1BBL (**Figure 10A and 10C**). It was also interesting that there was proliferative synergy between CD86 and 4-1BBL. Significantly, robust expansion of γδ T cells was observed in hypoxia in multiple scenarios, which corroborated the approach to expand hypoxia-sensitive γδ T cells. Culture conditions could be adapted to result in emergence of T cells that produced IL-17 and IFNγ (**Figure 10B and 10D**). The most IL-17 was observed with IL-21/4-1BBL in hypoxia and with IL-2/IL-21/4-1BBL in normoxia. The same conditions led to IFNγ production with the additional condition of IL-2/IL-21/CD86/4-1BBL in normoxia. It is also interesting that the absence of oxygen or lack of IL-2 abrogated the functionality of cells stimulated with CD86/4-1BBL. Moreover, IL-17 production in normoxia with IL-2/IL-21 was halted with the addition of CD86 but had no effect on IFNγ (**Figure 10D, left side, top and bottom panels**). It appears that 4-1BBL is the crucial molecule on aAPC to drive γδ T-cell proliferation and to yield IL-17 producing lineages, especially in normoxia. It was also unexpected that ICOS-L neither induced considerable growth nor led to IL-17 production. Nonetheless, the culture system can be adapted to generate γδ T cells that can produce IFNγ, IL-17, or both.

**The long-term objective** of this study was to create novel T cell-based treatments of disease-resistant ovarian cancer that can function in the hostile tumor environment with minimal toxicity to normal tissues. Our ROR1

chimeric antigen receptor (CAR) T-cells kill OvCa cells in both hypoxia and normoxia. Our expanded human γδ T cells killed OvCa tumor cells in vitro and in a mouse model.

Human γδ T cells expansion have historically been limited to the Vδ2[+] subset. Our ability to expand multiple subsets[42 (see also 47, 48)], and our data on IFNγ and IL-17 cytokines release and cell killing, and classical HLA *independence*, presents an opportunity to evaluate our expanded γδ T cells for **Ebola virus** and other emerging viral therapies. Anderson and Gustafsson's labs[47, 49, 50, 51], further report that γδ T cells using CD16 (FcγRIII) detect and phagocytose IgG coated bacteria, viruses, and antibody coated human cells, and furthermore be professional antigen presenting cells though HLA class I to CD8 and HLA class II to CD4 αβT-cells, that is, to act as "γδ T-APCs". A transgenic expression of a chimeric CAR-like "CD16/γ" consisting of FcγRIIIa (allotype V158) and the transmembrane and cytoplasmic domain of FcεRIγ on conventional CD4 and CD8 αβT-cells had been published[52], but the presence of CD16 on human γδ T cells and functionality in antibody dependent cellular cytotoxicity (ADCC), should enable use of our (and Anderson and Gustafsson's labs[47]) expanded γδ T cells with the rich repertoire of anti-cancer and anti-viral therapeutic monoclonal antibodies. We have already published a review "Clinical applications of gamma delta T cells with multivalent immunity"[55].

## KEY RESEARCH ACCOMPLISHMENTS

Major advances have been made on this study. There has been significant headway in targeting OvCa with both CAR^{+} T cells and with polyclonal γδ T cells.

- ROR1 CAR T-cells kill ROR1+ OvCa cancer cells.
- γδ T cells under hypoxia can proliferate with similar kinetics as in normoxia.
- γδ T cells produce both IFNγ and IL-17. Our expanded human γδ T cells killed OvCa tumor cells in vitro and in a mouse model.
- We are now able to routinely massively expand, for the first time[42 (see also 47, 48)], multiple γδ T cells subsets in culture with our aAPC that express CD64, CD86, CD137L (4-1BBL) and membrane-bound form of IL15.
- Wierda and Cooper have presented to NIH RAC a proposal for using ROR1 CAR T-cells to treat CLL[53].
- Weirda and Cooper opened (1/2015) the ROR1 CAR T-cell clinical trial at M.D. Anderson Cancer Center[54].

## CONCLUSION

We provide two T cell-based approaches for OvCa treatment that are both directly applicable to the clinic.

First, ROR1 was shown to be expressed on OvCa in both hypoxia and normoxia, and CAR^{+} T cells re-directed to ROR1 could specifically and efficiently lyse OvCa targets. *The ROR1 CAR T-cell clinical trial* NCT02194374 *is now open at M.D. Anderson Cancer Center.*

Second, polyclonal γδ T cells were expanded to clinically-relevant numbers on aAPC, could lyse many OvCa cell lines, and eliminated OvCa xenografts *in vivo*. Thus, translation of these T cell therapies will give women with advanced OvCa novel options in their treatment. γδ T cells are HLA-unrestricted and rapid expansion with our novel aAPCs may also have utility as off the shelf therapy for Ebola and other virus and bacteria mediated outbreaks.

## PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS

Publications

Deniger, DC 2013 T-cell treatments for solid and hematological tumors. Ph.D. Dissertation, *UT GSBS Dissertations and Theses (Open Access).* Paper 377.
http://digitalcommons.library.tmc.edu/utgsbs_dissertations/377

Deniger DC, Maiti S, Switzer KC, Mi T, Ramachandran V, Hurton LV, Ang S, Olivares S, Rabinovich B, Huls H, Lee DA, Bast RC, Jr., Champlin RE, Cooper LJN. Artificial antigen presenting cells propagate polyclonal gamma delta T cells with broad anti-tumor activity. *Clinical Cancer Res* 20: 5708-19. doi: 10.1158/1078-0432.CCR-13-3451. http://www.ncbi.nlm.nih.gov/pubmed/24833662.

Deniger DC, Switzer K, Mi T, Hurton L, Singh H, Huls H *et al.* Bi-specific T cells expressing polyclonal repertoire of endogenous gamma delta T-cell receptors and introduced CD19-specific chimeric antigen receptor. *Molecular Therapy* 2012; 21(3):638-47. http://www.ncbi.nlm.nih.gov/pubmed/23295945. doi: 10.1038/mt.2012.267.

Deniger DC, Moyes JS, Cooper LJ. Clinical applications of gamma delta T cells with multivalent immunity. *Front Immunol*. 2014; 5:636. doi: 10.3389/fimmu.2014.00636. PubMed PMID: 25566249; PubMed Central PMCID: PMC4263175.

Zhang M, Maiti S, Bernatchez C, Huls H, Rabinovich B, Champlin RE, Vence LM, Hwu P, Radvanyi L, Cooper LJ. A new approach to simultaneously quantify both TCR α- and β-chain diversity after adoptive immunotherapy. *Clin Cancer Res* 2012. 18: 4733-42. doi: 10.1158/1078-0432.CCR-11-3234. PMID: 22761473; PMCID: PMC3823368. (DTEA NanoString assay project was partially funded by Department of Defense).

Presentations

Deniger DC, Maiti S, Switzer K, Mi T, Hurton L, Singh H, Huls H, Olivares S, Cooper LJN. Gamma Delta T-Cells: Natural Tumor Killers Amplified by Chimeric Antigen Receptors. Society of Immunotherapy for Cancer (SITC) 27th Annual Meeting, Bethesda, MD, October 2012.

Wierda W, Cooper LJN, Kipps TJ. A Study to infuse ROR1 specific autologous T cells for patients with CLL. NIH RAC Review (Dec 4-5 2012). http://osp.od.nih.gov/sites/default/files/1192_Cooper.pdf

Deniger DC, Kipps TH, Olivares S, Singh H, Maiti S, Hurton LV, Switzer KC, Mi T, Thokala R, Huls H, Wierda WG, Cooper LJN Clinical Implications of ROR1-Specific T Cells That Target B-Cell Leukemia. Oral Presentation 502. Travel award to D.C. Deniger. American Society of Gene & Cell Therapy (ASGCT) 16th Annual Meeting, Salt Lake City, May, 2013.

## INVENTIONS, PATENTS AND LICENSES

Nothing to report.


## REPORTABLE OUTCOMES

We have accomplished the following:

- ROR1 CAR specific T-cells massive expansion in on our ROR1+ artificial antigen presenting cells (aAPC) culture.
- ROR1 CAR specific T-cells kill ovarian cancer cells (OvCa) in culture and in mouse models.
- γδ T cells Vδ1, Vδ2, and Vδ3 subsets were massively expanded in culture for the first time on our novel aAPCs.
- Comprehensive γδ T cells subset quantitation, for the first time, using specific mRNA counting on our direct T cell expression array (DTEA)[46] (Figure 6d).
- We published the utility of our massively expanded γδ T cells for leukemia in Deniger *et al* (2014)[42] (see also similar work published together by another lab [47], and commentary[48]).


## OTHER ACHIEVEMENTS

Drew C. Deniger successfully defended his Ph.D. dissertation[41], briefly continued as a postdoctoral fellow with us briefly, before transitioning to NIH/NCI to join the laboratory of Dr. Steven Rosenberg, a key researcher in

cellular immunotherapies. Deniger et al, "Clinical implications of ROR1-specific T cells that target B-cell leukemia", is in preparation43. Dr. Deniger and Cooper are coauthors on manuscripts on pancreatic cancer[44], and CD123 specific CAR T-cells to treat acute myelogenous leukemia (AML)[45].

Weirda et al[54] opened clinical trial NCT02194374 here at MD Anderson Cancer Center

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**Appendix 1. Schematics of CARs used in studies.** Chimeric antigen receptors (CAR) are fusion proteins of (i) antigen-specific binding regions of monoclonal antibodies constructed into single chain variable fragments (scFv), (ii) a hinge region, (iii) an IgG4 constant region (Fc) stalk, (iv) CD28 transmembrane (TM) domain, (v) co-stimulation domain (either CD28 (yellow) or CD137 (blue)), and CD3-zeta T cell signaling domains. ROR1-specific CARs signaling through CD28 (ROR1RCD28; Left) and CD137 (ROR1RCD137; Right).
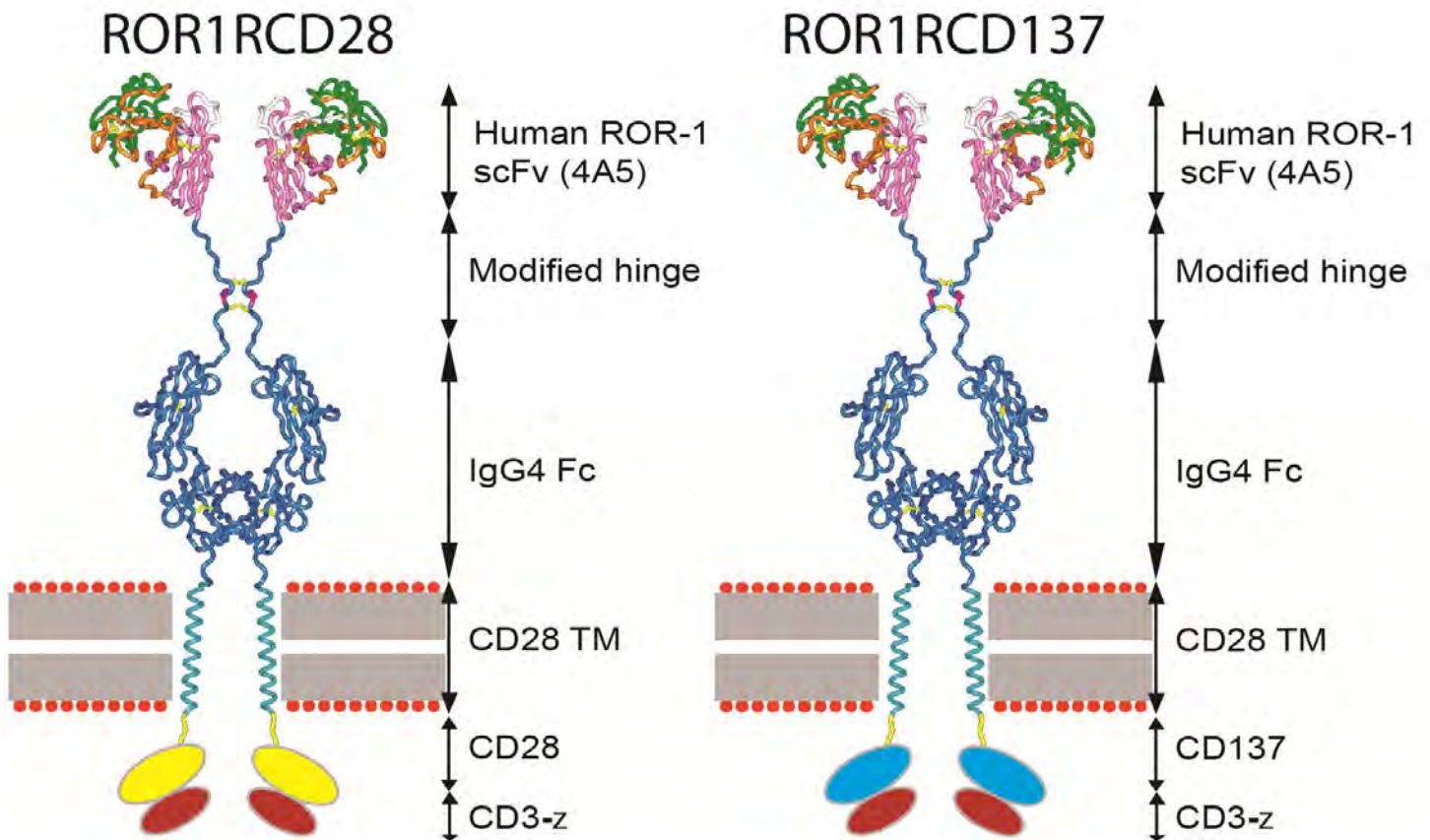
**Appendix 2. Schematic for propagation of CAR+ T cells.** Peripheral blood mononuclear cells (PBMC) were electroporated with *Sleeping Beauty* (SB) plasmids expressing SB transposon (CAR) and SB transposase by Lonza Amaxa nucleofection. Co-culture of CAR+ T cells with artificial antigen presenting cells (aAPC; "Clone1") led to numeric expansion of CAR+ T cells and enforced CAR expression through ROR1.
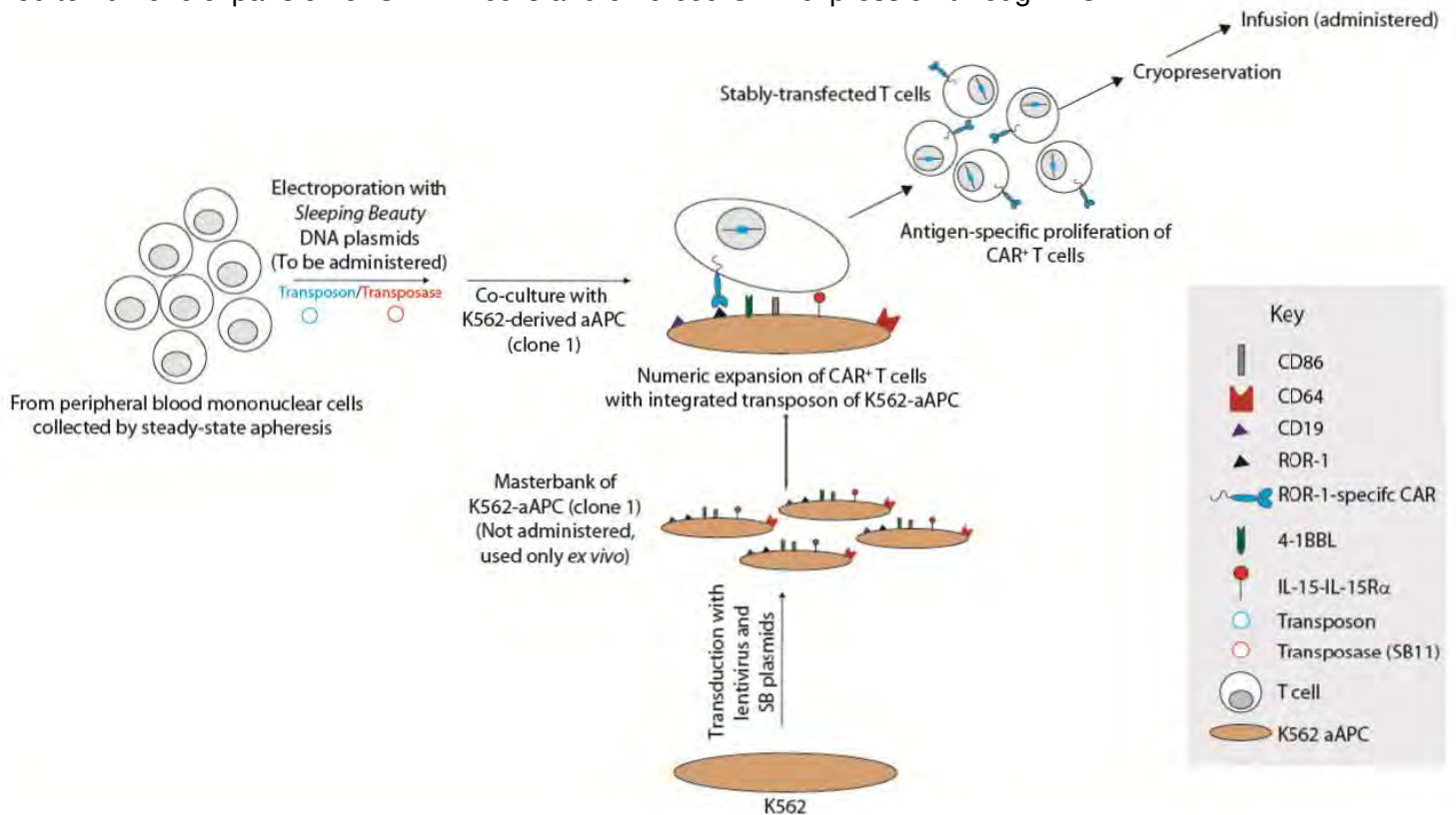
**Appendix 3. Phenotype of Clone1 aAPC used for propagation of ROR1-specific CAR$^+$ T cells.** Flow cytometry was used to assess recombinant expression of antigens (CD19 and ROR1), membrane-bound cytokines (IL-15 fused to IL-15R$\alpha$), co-stimulatory molecules (CD86 and CD137L), and Fc receptors (CD32 and CD64) on Clone1 aAPC. Isotype staining represented in open plots and filled plots represent antigen-specific staining.
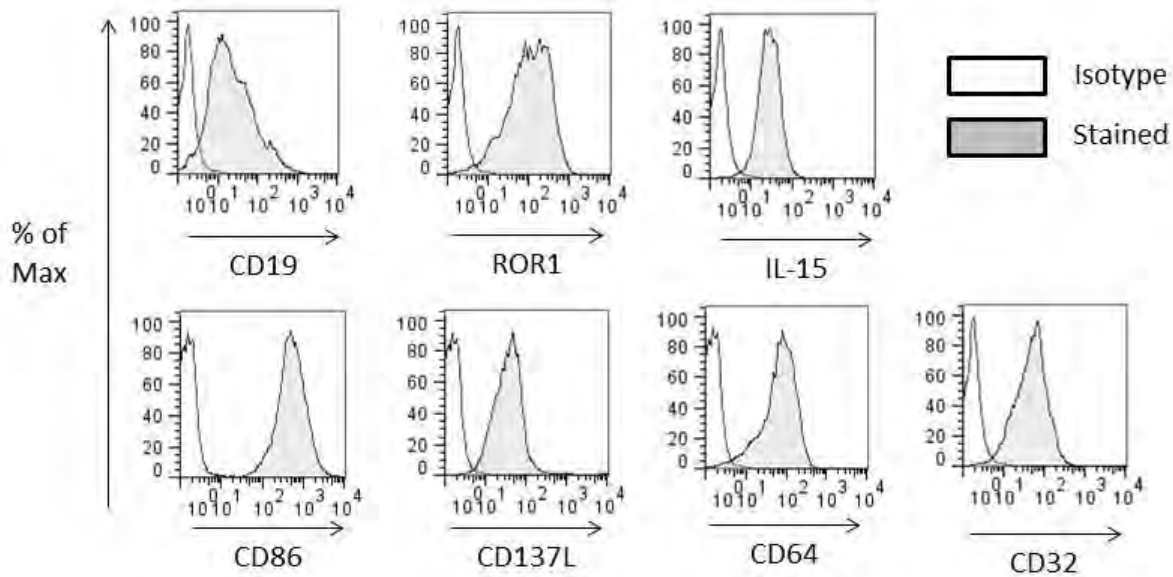
**Figure 1. ROR1 expression on OvCa in hypoxia and normoxia.** OvCa cell lines were grown in normoxia (20% $O_2$) or hypoxia (1% $O_2$) for three days at 37°C and were then stained for ROR1 expression with 4A5 monoclonal antibody specific for ROR1.[12] (A) Representative flow plots from A2780, EFO27, and OC314 cell lines where black lines are normoxia, red lines are hypoxia, dashes are isotype controls, and solid lines are ROR1 staining. (B) Normalized ROR1 (ROR1$_{normal}$) mean fluorescence intensity (MFI) in OvCa cell lines in normoxia (open bars) and hypoxia (closed bars). MFI was calculated by: MFI$_{ROR1}$ − MFI$_{isotype}$.
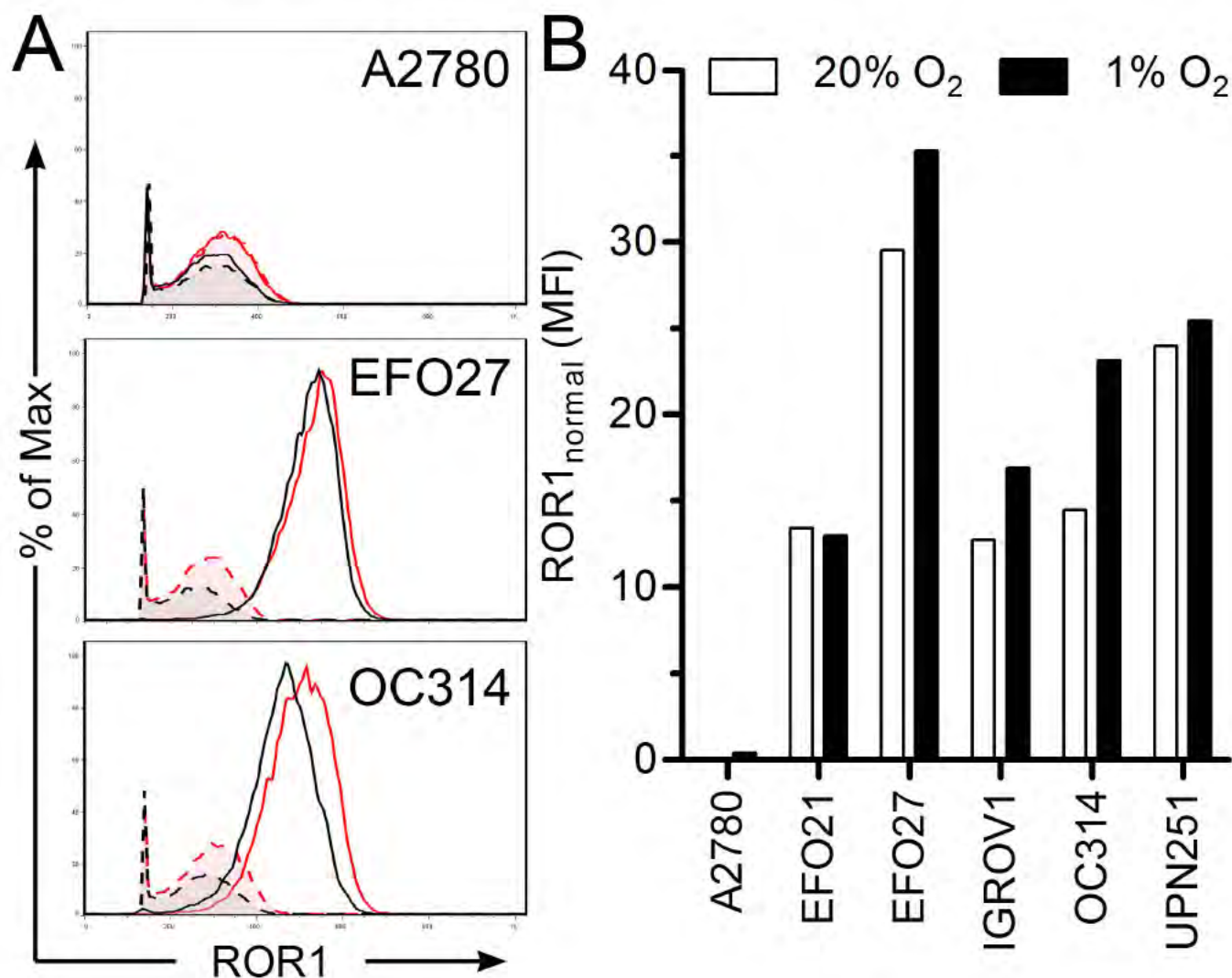
**Figure 2. Expansion of CAR+ T cells on ROR1+ aAPC.** PBMC were electroporated with ROR1-specific CARs signaling through CD28 (ROR1RCD28; closed squares) or CD137 (ROR1RCD137; open squares) and stimulated five times (each point on graphs) with ROR1+ aAPC along with IL-2 and IL-21. (A) Total cell counts during culture period. (B) CAR+ T cell counts during culture period.
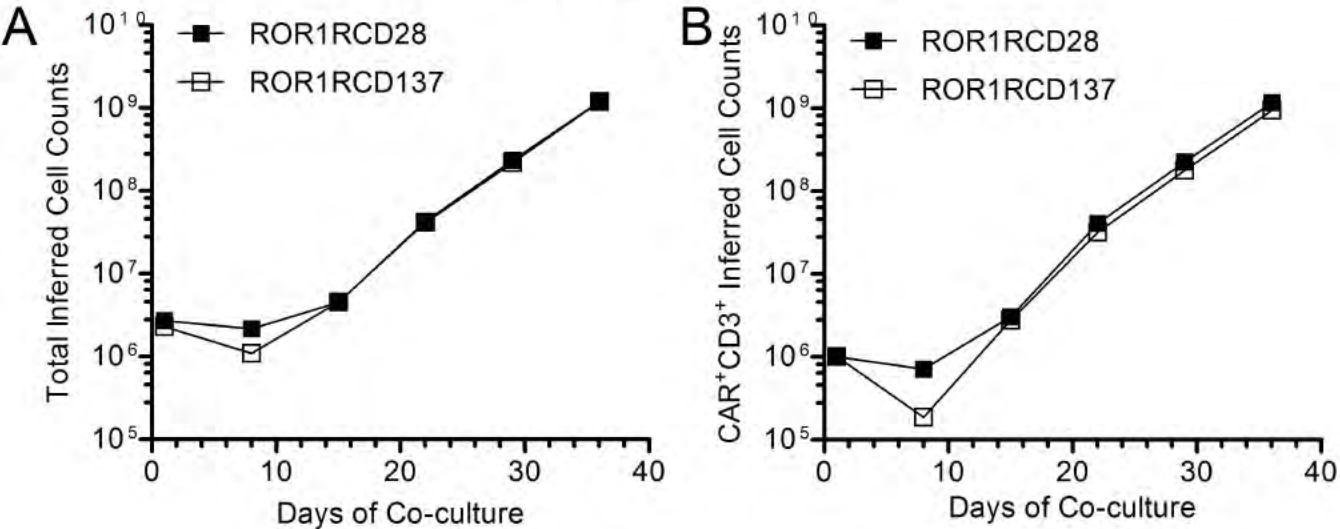
**Figure 3. CAR expression after expansion on ROR1⁺ aAPC.** Stable CAR expression after 29 days of co-culture on ROR1⁺ aAPC. Sham electroporated "No DNA" cells were stimulated with OKT3-loaded aAPC for negative controls. Gate frequencies are displayed in upper right corners.
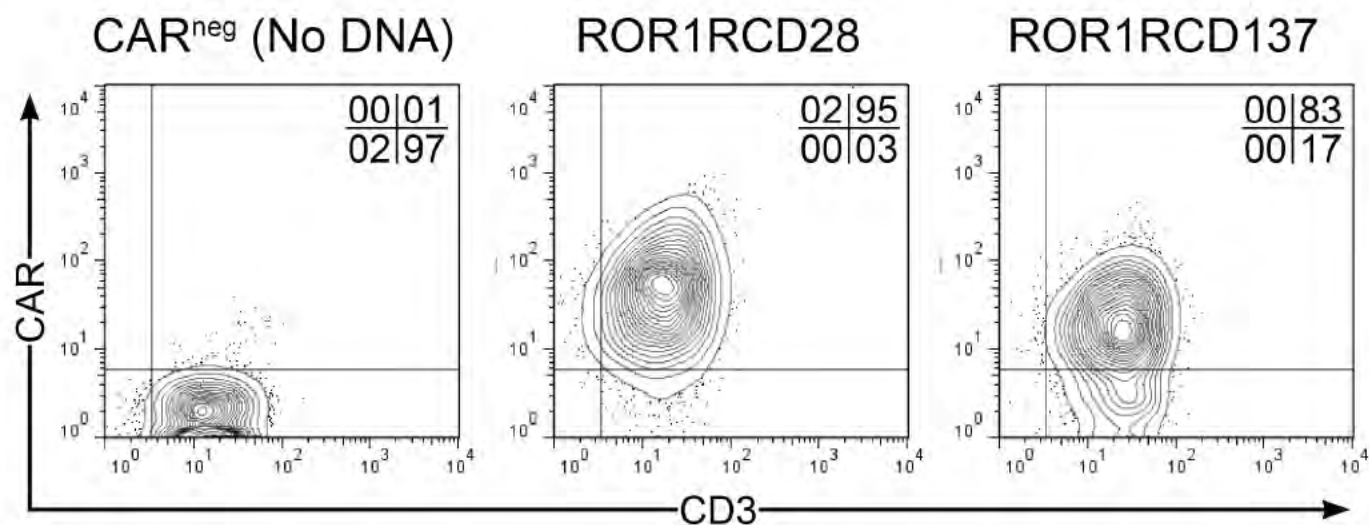
**Figure 4. Specific killing of ROR1+ tumor cells by CAR+ T cells.** Standard 4-hour chromium release assays were performed after 35 days of expansion on aAPC with CAR$^{neg}$ (triangles), ROR1RCD28 (circles), or ROR1RCD137 (squares) T cells against (A) EL4-ROR1$^{neg}$, (B) EL4-ROR1$^{+}$, (C) A2780 (ROR1$^{neg}$ OvCa), and (D) EFO27 (ROR1$^{+}$ OvCa) cell lines. Two-way ANOVA with Bonferroni's post-tests used for statistical analyses on triplicate measurements where *$p < 0.05$ and ***$p < 0.001$.
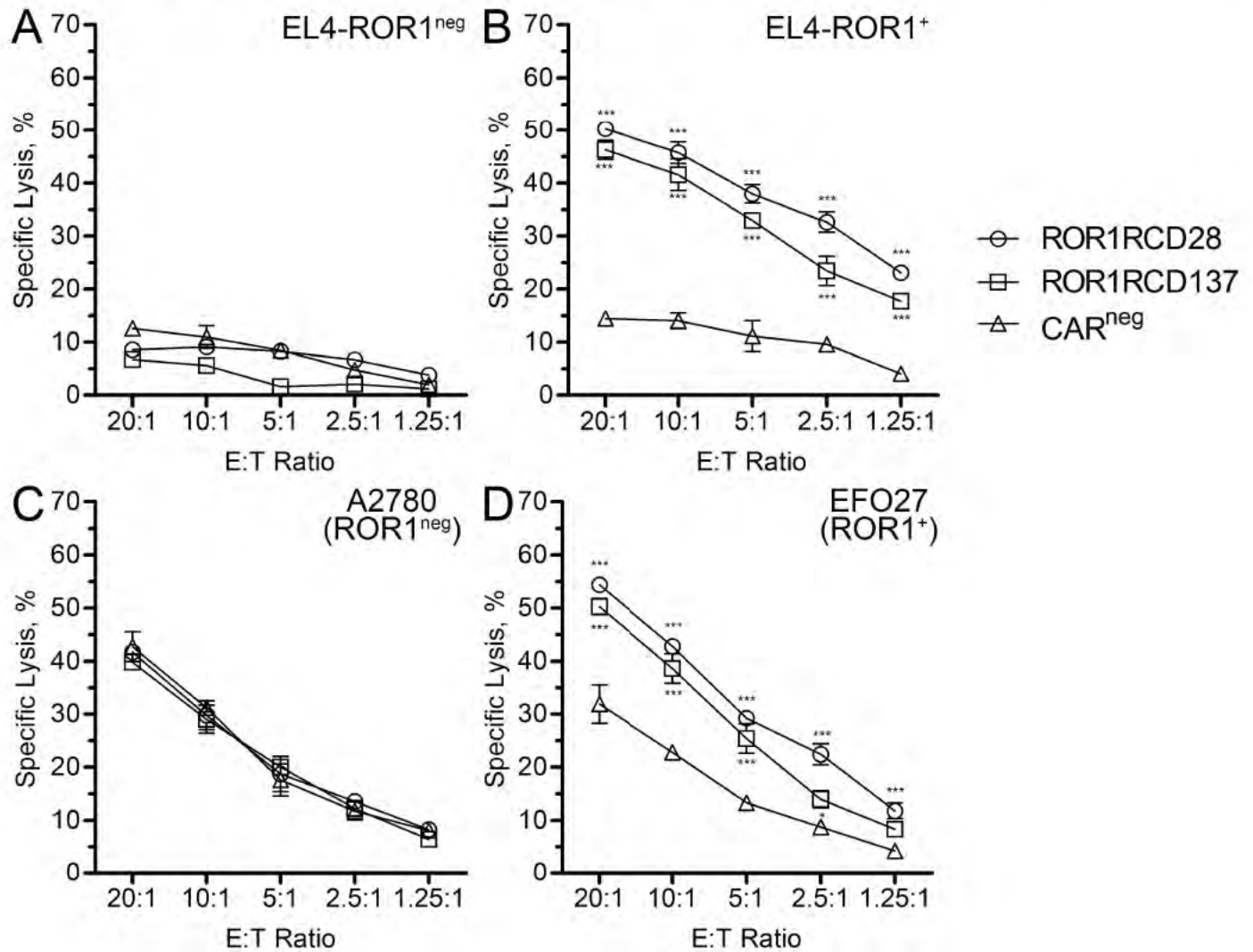
**Figure 5. Expansion of γδ T cells on aAPC.** (A) Flow cytometry of CD3 (y-axis) and TCRγδ (x-axis) expression in PBMC prior to paramagnetic bead sorting at Day 0 and of T cell cultures after 22 days of co-culture on aAPC. One representative donor of four healthy donors is shown and quadrant gate frequencies are displayed in the upper right corners of flow plots. (B) Total inferred cell counts of viable cells during co-culture period. (C) Fold expansion of cells in co-culture during co-culture period. Black lines are mean ± SD from 4 healthy donors, gray lines are individual donors, arrows represent stimulations with aAPC, and data are pooled from two independent experiments.
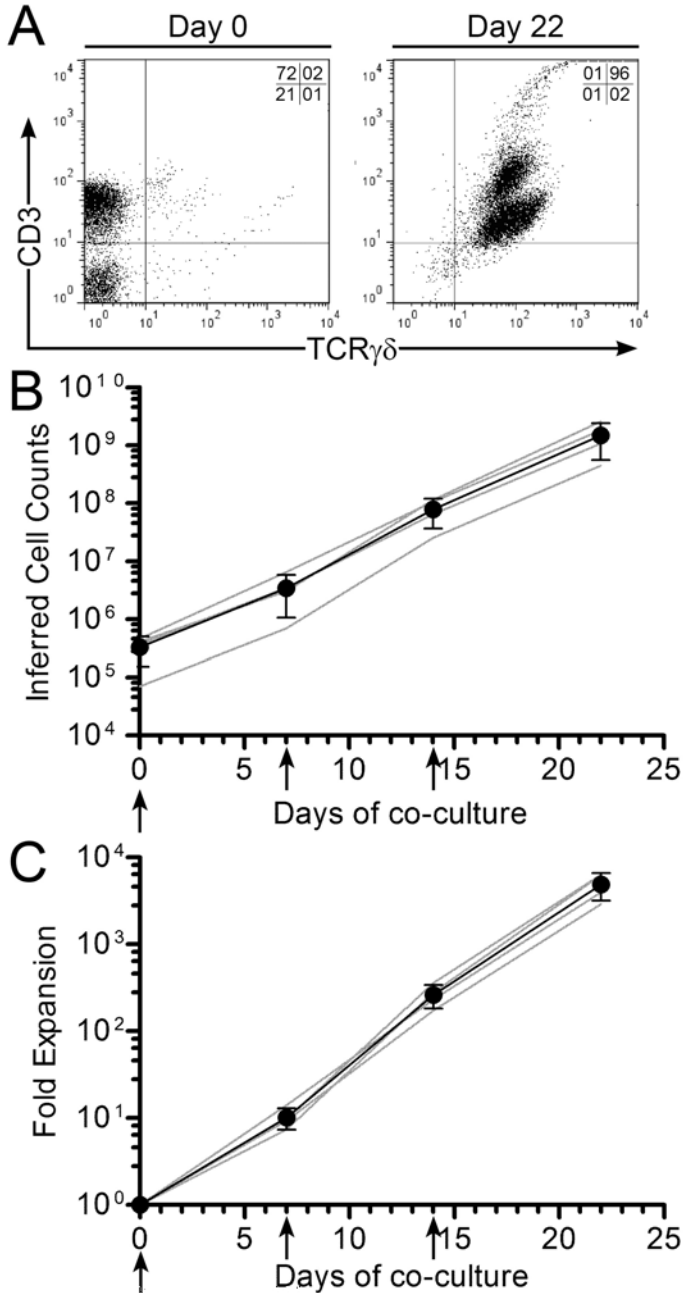
**Figure 6. TCRγδ expression on aAPC-expanded γδ T cells.** (A) Flow cytometry of TCRδ2 and TCRδ1 expression in γδ T cells at day 22 of co-culture. (B) Flow cytometry of TCRδ2 and TCRγ9 expression in γδ T cells at day 22 of co-culture. Numbers in lower right corners correlate with donor numbers in (A) and (B) where quadrant frequencies are displayed in upper right corners. (C) Direct TCR expression array "DTEA" detection of Vδ mRNA alleles in γδ T cells at day 22 of co-culture where Vδ1*01, Vδ2*02, and Vδ3*01 alleles are in graphs from left to right, respectively, and each of the four donors are numbered on x-axes. (D) DTEA detection of Vγ allele mRNA expression in γδ T cells at day 22. Lines are mean ± SD where each circle represents an individual healthy donor grown in two independent experiments.
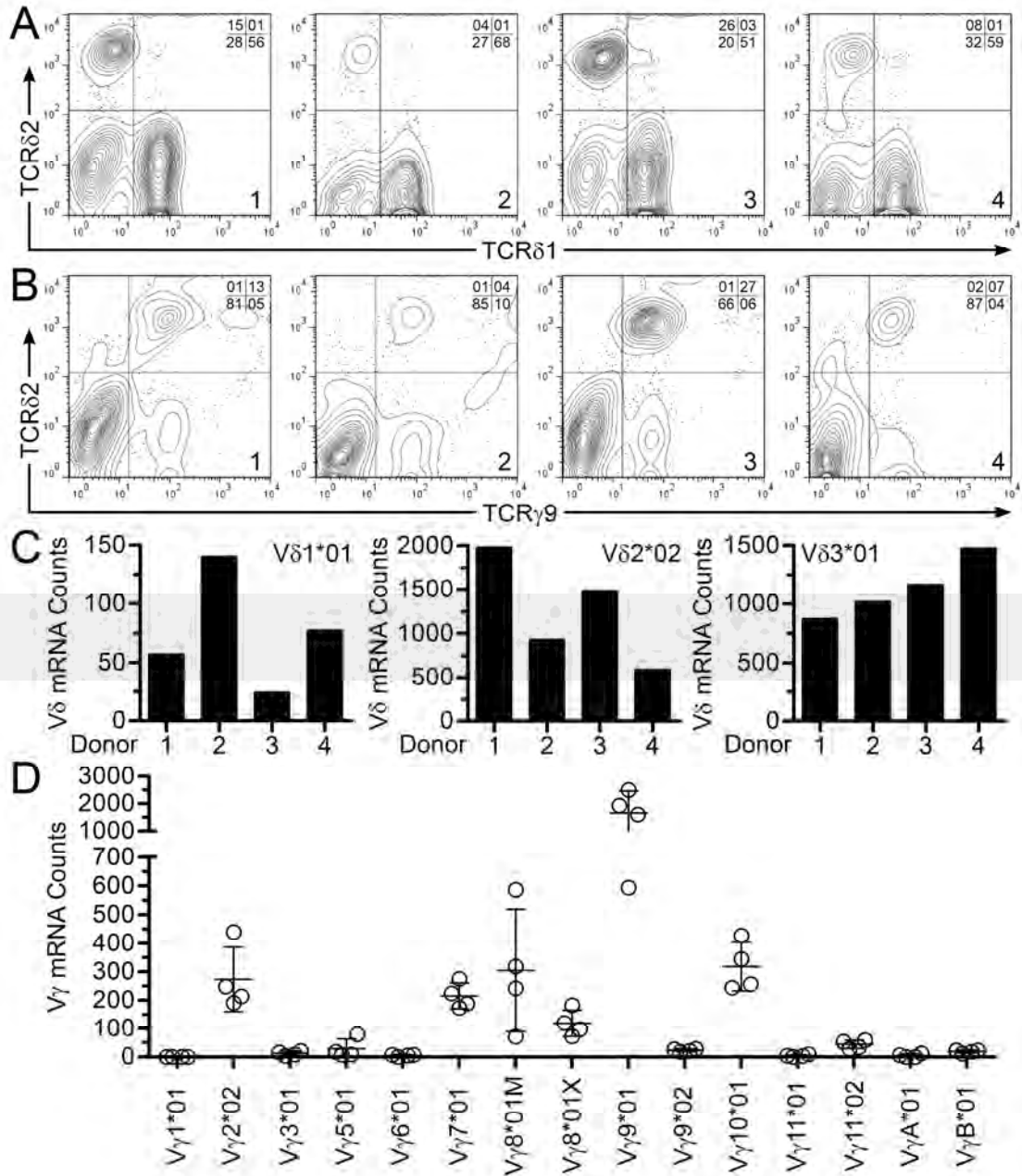
**Figure 7. Cytokines and chemokines secreted by γδ T cells expanded on aAPC.** Cells at day 22 were co-cultured with a mock activation cocktail (complete media) or leukocyte activation cocktail (LAC; PMA/Ionomycin) for 6 hours at 37°C. Conditioned media was interrogated on 27-Plex Luminex array to detect cytokines and chemokines. A, Th2 cytokines. B, Th17 cytokines. C, Th1 cytokines. D, Chemokines. Data are mean ± SD from 4 healthy donors. Student's t-test performed for statistical analysis between mock and LAC groups for each molecule. *$p<0.05$, **$p<0.01$, and ***$p<0.001$
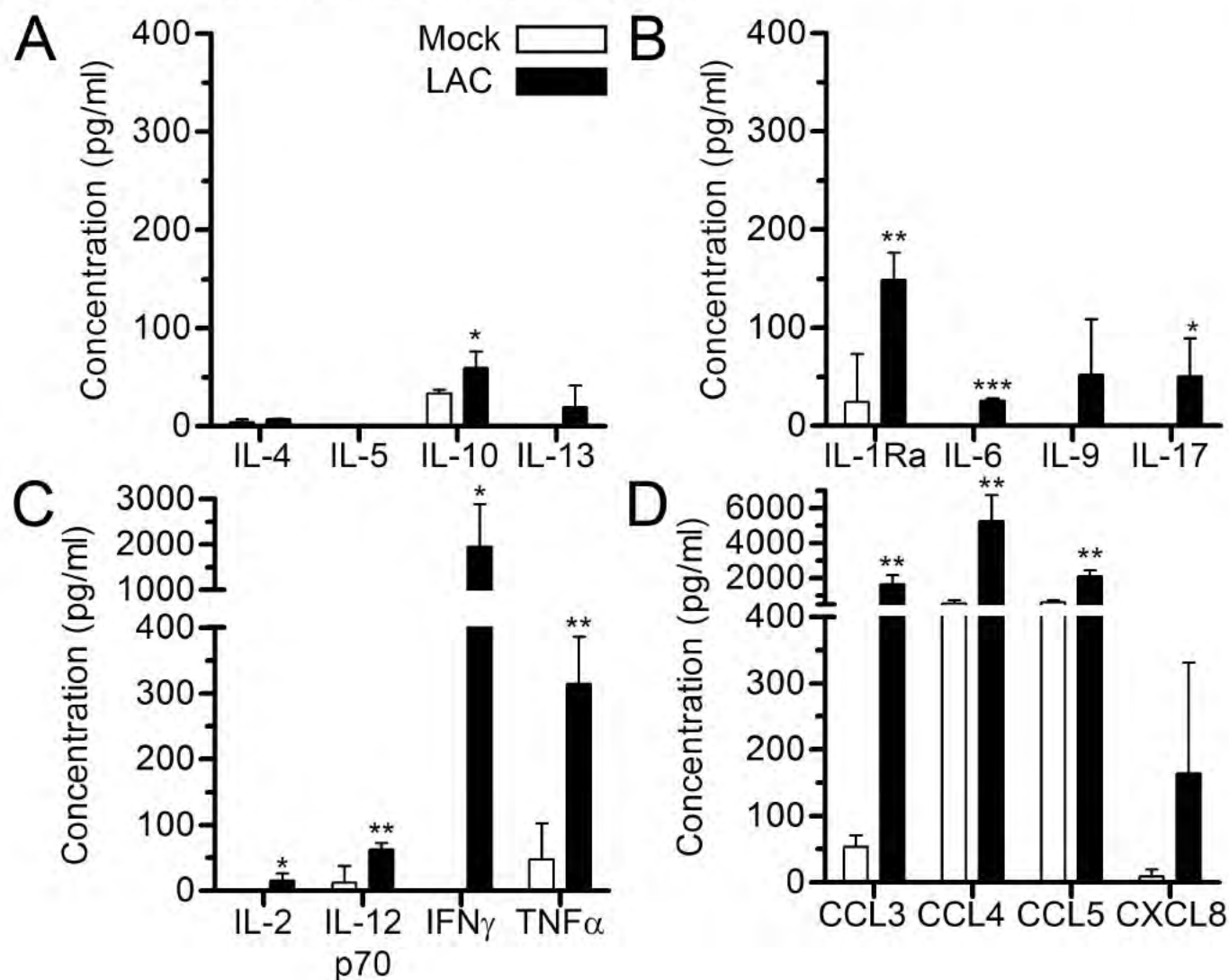
**Figure 8. *In vitro* cytolysis of tumor cells by γδ T cells.** Standard 4-hour chromium release assays were performed with increasing effector (γδ T cells) to target (E:T) ratios against healthy B cells from an allogeneic donor (top left; one of four representative donors) and OvCa cell lines A2780, EFO21, EFO27, IGROV1, OC314, UPN251, and CAOV3. Each line represents an individual effector where data is mean ± SD (n = 3 wells per assay) from two independent experiments.
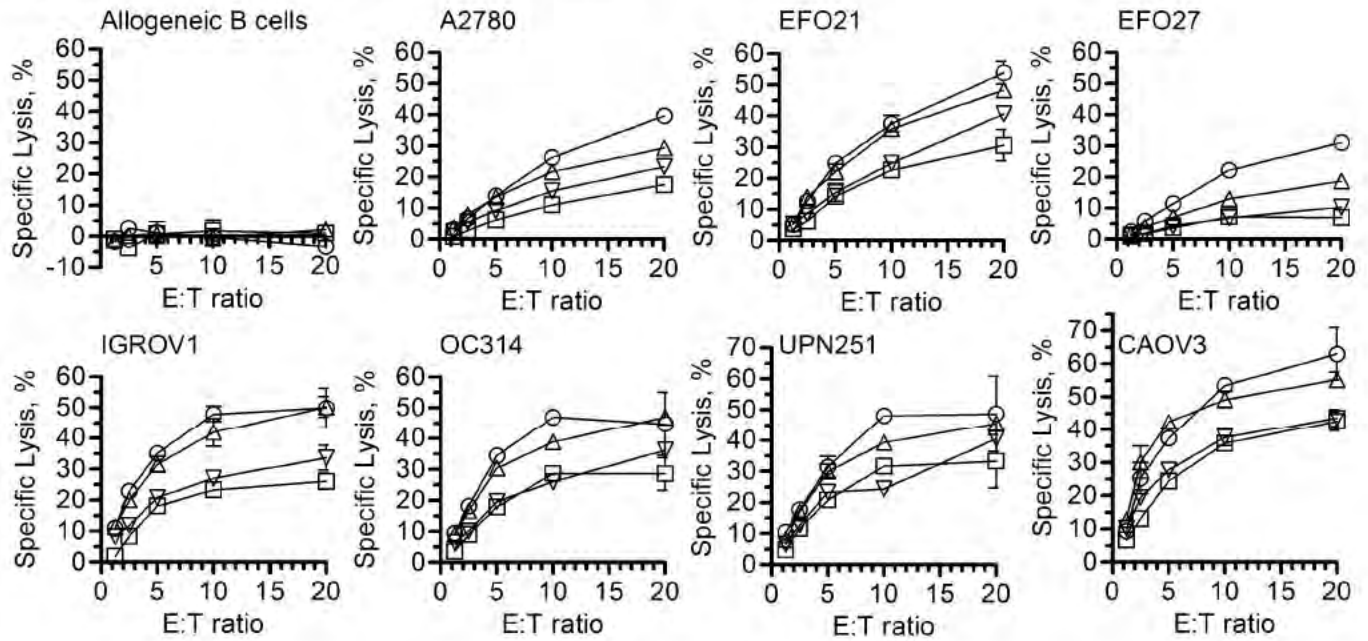
**Figure 9. OvCa tumor clearance by polyclonal $\gamma\delta$ T cells *in vivo*.** (A) Schematic of experiment with legend to right. (B) Kinetics of bioluminescent flux of CAOV3-*ffLuc* xenografts from mock treated (closed squares) and $\gamma\delta$ T cell treated (open squares) mice during experiment. Two-way ANOVA with Bonferroni's post-tests was used for statistical analysis where n = 10, *$p<0.05$, and ***$p<0.001$. (C) Representative images 43 days after engraftment and treated with PBS (top panels) or $\gamma\delta$ T cells (bottom panels). (D) Long-term BLI flux comparison between day prior to treatment (Day 7; squares) and 79 days (circles) post-engraftment. Student's t-test performed between time points for each group with p-values above comparisons.
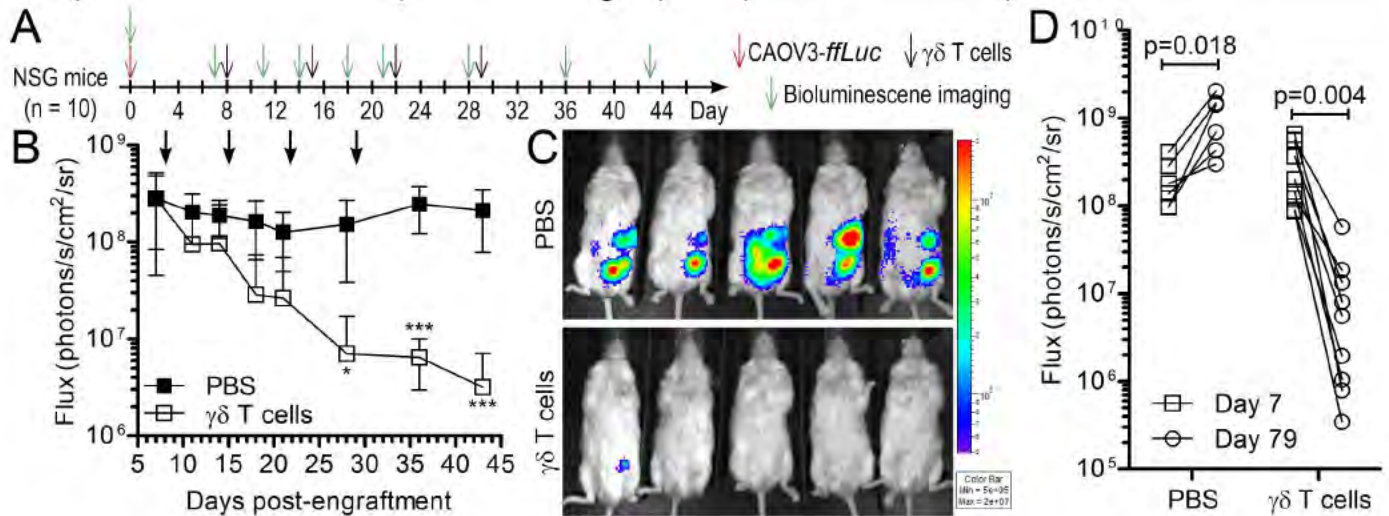
**Figure 10. $\gamma\delta$ T cells expanded on aAPC in normoxia and hypoxia and polarized towards IFN$\gamma$ and IL-17 production.** PBMC were sorted on TCR$\gamma$/$\delta$+ isolation kit and $10^4$ purified $\gamma\delta$ T cells were then given a single stimulation with either IL-21 (A and B) or IL-2 and IL-21 (C and D) along with no aAPC or $2\times10^4$ aAPC with one of the following co-stimulatory molecules: (i) none, (ii) CD70, (iii) CD86, (iv) 4-1BBL, (v) ICOS-L, or (vi) CD86 and 4-1BBL. Duplicate cultures were then placed in 20% $O_2$ or 1% $O_2$ for nine days at 37$^o$C with humidified conditions. (A and C) After the 9 day incubation, cells were counted using trypan blue exclusion. Numbers above hatched bars represent fold changes of cytokine-treated cells compared to cultures without cytokines (bars not hatched). (B and D) Remaining cells were then mock activated (black line) or activated with leukocyte activation cocktail (LAC, PMA/Ionomycin, red line) for 6 hours in the presence of secretory inhibitor GolgiPlug at the same oxygen concentration as the 9 day culture. Cells were then stained for CD3, TCR$\gamma\delta$, IFN$\gamma$, and IL-17 and analyzed by flow cytometry. Data shown are histograms gated on CD3$^+$TCR$\gamma\delta^+$ cells where cytokine detected (x-axis) is in upper right corner and y-axis is % of maximum value. Top panels are 4-1BBL cultures and bottom panels are CD86/4-1BBL cultures. Left panels are 20% $O_2$ and right panels are 1% $O_2$.
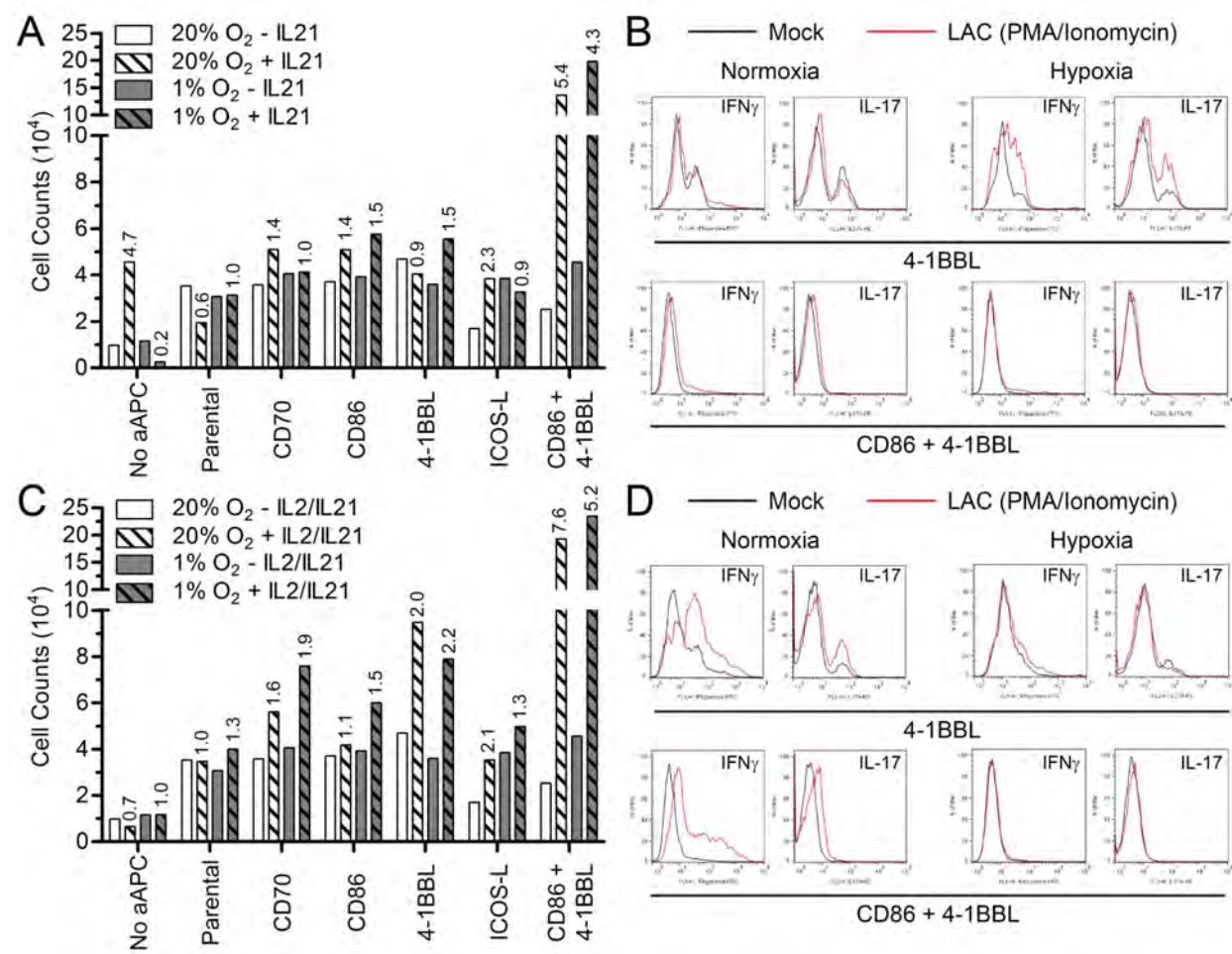
**ATTACHMENT**

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# Clinical Cancer Research

# Activating and Propagating Polyclonal Gamma Delta T Cells with Broad Specificity for Malignancies

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*Cancer Therapy: Preclinical*
See related commentary by Déchanet-Merville, p. 5573

# Activating and Propagating Polyclonal Gamma Delta T Cells with Broad Specificity for Malignancies

Drew C. Deniger[1,5], Sourindra N. Maiti[1], Tiejuan Mi[1], Kirsten C. Switzer[1], Vijaya Ramachandran[2], Lenka V. Hurton[1,5], Sonny Ang[1], Simon Olivares[1], Brian A. Rabinovich[1], M. Helen Huls[1], Dean A. Lee[1,5], Robert C. Bast Jr[3], Richard E. Champlin[4], and Laurence J.N. Cooper[1,5]

## Abstract

**Purpose:** To activate and propagate populations of $\gamma\delta$ T cells expressing polyclonal repertoire of $\gamma$ and $\delta$ T cell receptor (TCR) chains for adoptive immunotherapy of cancer, which has yet to be achieved.

**Experimental Design:** Clinical grade artificial antigen presenting cells (aAPC) derived from K562 tumor cells were used as irradiated feeders to activate and expand human $\gamma\delta$ T cells to clinical scale. These cells were tested for proliferation, TCR expression, memory phenotype, cytokine secretion, and tumor killing.

**Results:** $\gamma\delta$ T cell proliferation was dependent upon CD137L expression on aAPC and addition of exogenous IL2 and IL21. Propagated $\gamma\delta$ T cells were polyclonal as they expressed *TRDV1*, *TRDV2 2*, *TRDV3*, *TRDV5*, *TRDV7*, and *TRDV8* with *TRGV2*, *TRGV3F*, *TRGV7*, *TRGV8*, *TRGV9\*A1*, *TRGV10\*A1*, and *TRGV11* TCR chains. IFN$\gamma$ production by V$\delta$1, V$\delta$2, and V$\delta$1$^{neg}$V$\delta$2$^{neg}$ subsets was inhibited by pan TCR$\gamma\delta$ antibody when added to cocultures of polyclonal $\gamma\delta$ T cells and tumor cell lines. Polyclonal $\gamma\delta$ T cells killed acute and chronic leukemia, colon, pancreatic, and ovarian cancer cell lines, but not healthy autologous or allogeneic normal B cells. Blocking antibodies demonstrated that polyclonal $\gamma\delta$ T cells mediated tumor cell lysis through combination of DNAM1, NKG2D, and TCR$\gamma\delta$. The adoptive transfer of activated and propagated $\gamma\delta$ T cells expressing polyclonal versus defined V$\delta$ TCR chains imparted a hierarchy (polyclonal>V$\delta$1>V$\delta$1$^{neg}$V$\delta$2$^{neg}$>V$\delta$2) of survival of mice with ovarian cancer xenografts.

**Conclusions:** Polyclonal $\gamma\delta$ T cells can be activated and propagated with clinical grade aAPCs and demonstrate broad antitumor activities, which will facilitate the implementation of $\gamma\delta$ T cell cancer immunotherapies in humans. *Clin Cancer Res; 20(22); 5708 19. ©2014 AACR.*

## Introduction

Human $\gamma\delta$ T cells exhibit an endogenous ability to specifically kill tumors and hold promise for adoptive immunotherapy. They have innate and adaptive qualities exhibiting a range of effector functions, including cytolysis upon cell contact (1, 2). Recognition and subsequent killing of tumor is achieved upon ligation of antigens to hetero dimers of $\gamma$ and $\delta$ T cell receptor (TCR) chains. The human TCR variable (V) region defines 14 unique V$\gamma$ alleles (*TRGV*), 3 unique V$\delta$ alleles (*TRDV1*, *TRDV2*, and *TRDV3*), and 5 V$\delta$ alleles that share a common nomenclature with V$\alpha$ alleles (*TRDV4/TRAV14*, *TRDV5/TRAV29*, *TRDV6/TRAV23*, *TRDV7/TRAV36*, and *TRDV8/TRAV38 2*; ref. 3). T cells expressing TCR$\alpha$/TCR$\beta$ heterodimers compose approximately 95% of peripheral blood T cells and recog nize peptides in the context of MHC (4). In contrast, TCR$\gamma\delta$ ligands are recognized independent of MHC and these cells are infrequent (1% 5% of T cells) in peripheral blood (1, 5, 6). Many conserved ligands for TCR$\gamma\delta$ are present on cancer cells, thus an approach to propagating these T cells from small starting numbers while maintaining a polyclon al repertoire of $\gamma\delta$ TCRs has appeal for human application.

Clinical trials highlight the therapeutic potential of $\gamma\delta$ T cells, but numeric expansion is needed for adoptive immu notherapy because they circulate at low frequencies in peripheral blood. Methods to propagate $\alpha\beta$ T cells, for e. g., using interleukin 2 (IL2) and/or antibody cross linking CD3, cannot sustain proliferation of $\gamma\delta$ T cells (7, 8). Aminobisphosphonates, for e.g., zoledronic acid, have been used to initiate a proliferative signal in $\gamma\delta$ T cells (5, 9), but only one lineage of $\gamma\delta$ T cells, expressing V$\gamma$9V$\delta$2 TCR, can be reliably expanded by zoledronic acid. The adoptive transfer of V$\gamma$9V$\delta$2 T cells has yielded clinical responses for investigational treatment of solid and hematologic cancers (10 14). Furthermore, long term remission of leukemia among recipients of haploidentical $\alpha\beta$ T cell depleted

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American Association for Cancer Research

## Translational Relevance

γδ T cells have anticancer activity, but only one subset, Vγ9Vδ2, has been harnessed for immunotherapy. Our study establishes that artificial antigen presenting cells (aAPC), IL2, and IL21 can activate and propagate γδ T cells with polyclonal T cell receptor repertoire to clinical scale. The heterogeneous population of γδ T cells produced from *ex vivo* culture secreted proinflammatory cytokines, lysed a broad range of malignancies, and improved survival in an ovarian cancer xenograft model. Given that γδ T cells are not thought to recognize ligands in the context of MHC, there is limited risk of graft versus host disease in an allogeneic setting. Thus, third party γδ T cells from an unrelated (healthy) donor could be produced in bulk and be administered as an off the shelf investigational therapy for hematologic and solid tumors. The aAPCs are already available as a clinical reagent, which will facilitate the human application of polyclonal γδ T cells.

hematopoietic stem cell transplantation (HSCT) correlated with increased frequency of engrafted donor derived Vδ1 cells (8, 15 17). However, direct administration of Vδ1 cells or other non Vγ9Vδ2 cell lineages has yet to be performed. In addition, no reports to date have described the therapeutic impact of Vδ1$^{neg}$Vδ2$^{neg}$ cells in cancer immunotherapy and this subset has not been directly compared with T cells expressing Vδ1 and Vδ2 TCRs. Thus, there are significant gaps in the knowledge and human application of non Vγ9Vδ2 lineages.

Given that γδ T cells have endogenous anticancer activity, such as against K562 cells (8, 18), we hypothesized that malignant cells would serve as a cellular substrate to propagate polyclonal γδ T cells. K562 cells have been genetically modified to function as artificial antigen presenting cells (aAPC) to *ex vivo* activate and numerically expand αβ T cells and NK cells (19 23). We determined that IL2, IL21, and γ irradiated K562 derived aAPCs (designated clone #4, genetically modified to coexpress CD19, CD64, CD86, CD137L, and a membrane bound mutein of IL15 (mIL15); used in selected clinical trials at MD Anderson Cancer Center, Houston, TX) can sustain the proliferation of γδ T cells with polyclonal TCR repertoire. Polyclonal γδ T cells exhibited broad tumor reactivity and displayed a multivalent response to tumors as evidenced by the ability of separated Vδ subpopulations to kill and secrete cytokine against the same tumor target. Furthermore, killing by polyclonal populations was multifactorial being mediated through DNAM1, NKG2D, and TCRγδ. Tumor xenografts were eliminated by both polyclonal and distinct γδ T cell subsets, and mice treated with polyclonal γδ T cells had superior survival. Given the availability of aAPCs as a clinical reagent, trials can, for the first time, evaluate polyclonal populations of γδ T cells as a cancer immunotherapy.

## Materials and Methods

### Cell lines

HCT 116, Kasumi 3, and K562 were acquired from ATCC. Jurkat was purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen. cALL 2 and RCH ACV were gifts from Dr. Jeff Tyner (Oregon Health & Science University, Portland, OR). BxPC 3, MiaPaCa 2, and Su8686 (pancreatic cancer) were donated by Dr. Vijaya Ramachan dran (MD Anderson Cancer Center). A2780, CAOV3, EFO21, EFO27, Hey, IGROV1, OAW42, OC314, OVCAR3, and UPN251 (ovarian cancer) were provided by Dr. Robert C. Bast, Jr. (MD Anderson Cancer Center, Houston, TX). Identities of all cell lines were confirmed by STR DNA Fingerprinting at MD Anderson Cancer Center's "Characterized Cell Line Core" and cells were used within 6 months of authentication.

### Propagation of γδ T cells

Peripheral blood mononuclear cells (PBMC) and umbilical cord blood (UCB) were isolated from healthy volunteers by Ficoll Hypaque (GE Healthcare) after informed consent (24). Thawed PBMCs ($10^8$) were initially treated with CD56 microbeads (cat# 130 050 401, Miltenyi Biotec) and separated on LS columns (cat# 130 042 401, Miltenyi Biotec) to deplete NK cells from cultures because they proliferate on aAPCs (23) and would contaminate the purity of the γδ T cell product. Unlabeled cells from CD56 depletion sorting were then labeled with TCRγ/δ+ T cell isolation kit (cat# 130 092 892, Miltenyi Biotec) and placed on LS columns to separate γδ T cells in the unlabeled fraction from other cells attached to magnet. γδ T cells were cocultured at a ratio of one T cell to two γ irradiated (100 Gy) aAPCs (clone #4) in presence of exogenous IL2 (Aldesleukin; Novartis; 50 IU/mL), and IL21 (cat# AF20021; Peprotech; 30 ng/mL) in complete media (CM; RPMI, 10% FBS, 1% Glutamax). Cells were serially restimulated with addition of γ irradiated aAPCs every 7 days for 2 to 5 weeks in presence of soluble cytokines, which were added three times per week beginning the day of aAPC addition. K562 were genetically modified to function as aAPCs (clone #4) as previously described (25, 26). Validation of coexpression of CD19, CD64, CD86, CD137L, and eGFP (IL15 peptide fused in frame to IgG4 Fc stalk and coexpressed with eGFP) on aAPC clone #4 was performed before addition to T cell cultures (25). Fluorescence activated cell sorting (FACS) was used to isolate Vδ1 (TCRδ1$^+$TCRδ2$^{neg}$), Vδ2 (TCRδ1$^{neg}$TCRδ2$^+$), and Vδ1$^{neg}$Vδ2$^{neg}$ (TCRδ1$^{neg}$TCRδ2$^{neg}$) populations, which were stimulated twice as above with aAPC clone #4, phenotyped, and used for functional assays. γδ T cells from UCB were isolated by FACS from thawed mononuclear cells using anti TCRγδ and anti CD3 monoclonal antibodies (mAb) and were stimulated for 5 weeks on aAPCs/cytokines as per PBMCs.

### Abundance and identity of mRNA molecules by DTEA

At designated times after coculture on aAPCs, T cells were lysed at a ratio of 160 μL RLT buffer (Qiagen) per $10^6$ cells and frozen at 80°C. RNA lysates were thawed and

immediately analyzed using nCounter Analysis System (NanoString Technologies) with "designer TCR expression array" (DTEA), as previously described (27, 28). DTEA data were normalized to both spiked positive control RNA and housekeeping genes (*ACTB*, *G6PD*, *OAZ1*, *POLR1B*, *POLR2A*, *RPL27*, *RPS13*, and *TBP*). Spiked positive control normalization factor was calculated from the average of sums for all samples divided by the sum of counts for an individual sample. Spiked positive control normalization factor was calculated from the average of geometric means for all samples divided by the geometric mean for an individual sample. Normalized counts were reported.

### Flow cytometry

Cells were phenotyped with antibodies detailed in Supplementary Table S1. Gating strategy is displayed in Supplementary Fig. S1. Samples were acquired on FACS Calibur (BD Biosciences) and analyzed with FlowJo software (version 7.6.3).

### Cytokine production and cytolysis assays

Expression of cytokines was assessed by intracellular staining and secretion of cytokines into tissue culture supernatants was evaluated by Luminex multiplex analysis. *In vitro* specific lysis was assessed using a standard 4 hour CRA, as previously described (25). Additional information can be found in the Supplementary Materials and Methods.

### Mouse experiments

*In vivo* antitumor efficacy was assessed in NSG mice (NOD.Cg Prkdc$^{scid}$Il2rγ$^{tm1Wjl}$/SzJ; Jackson Laboratories). CAOV3 ovarian cancer cell line was transduced with recombinant lentivirus (Supplementary Fig. S2) encoding mKate red fluorescence protein (29) to identify transduced cells and enhanced *firefly luciferase* (*effLuc*) for noninvasive bioluminescence imaging (30). CAOV3 *effLuc* mKate (clone 1C2; $3 \times 10^6$ cells/mouse) tumors were established by intraperitoneal injection and mice were randomly distributed into treatment groups. Eight days later (designated day 0), a dose escalation regimen was initiated with γδ T cells and PBS (negative control) administered intraperitoneally. T cell doses were $3 \times 10^6$, $6 \times 10^6$, $10^7$, and $1.5 \times 10^7$ on days 0, 7, 14, and 21, respectively. Noninvasive bioluminescence images (BLI) was performed during the course of the experiments to serially measure tumor burden of CAOV3 *effLuc* mKate following subcutaneous administration of D Luciferin (cat#122796, Caliper) as detected with IVIS 100 Imager (Caliper). BLI was analyzed using Living Image software (version 2.50, Xenogen, Caliper).

## Results

### Ex vivo numeric expansion of γδ T cells on aAPCs depends on costimulation and cytokines

The adoptive transfer of γδ T cells requires *ex vivo* propagation as starting numbers from PBMCs are limiting (gating on lymphocyte pool: 3.2% ± 1.2%; mean ± SD; n = 4; Fig. 1A). γδ T cells from PBMCs were isolated by "negative" paramagnetic bead selection and cocultured for 22 days

with weekly addition of γ irradiated K562 derived aAPCs (clone #4) in the presence of soluble recombinant IL2 and IL21 in alignment with protocols at MD Anderson Cancer Center (Houston, TX) for propagation of clinical grade αβ T cells. This resulted in the outgrowth of a population of T cells homogeneously coexpressing CD3 and TCRγδ (97.9% ± 0.6%). NK cells (CD3$^{neg}$CD56$^+$) and αβ T cells (TCRαβ$^+$) were absent from these cultures supporting the purity of the γδ T cell product. Populations of TCRδ1$^+$TCRδ2$^{neg}$, TCRδ1$^{neg}$TCRδ2$^+$, and TCRδ1$^{neg}$TCRδ2$^{neg}$ were detected indicating that aAPCs, IL2, and IL21 supported polyclonal γδ T cell proliferation (Fig. 1A far right). Cells were activated as marked by expression of CD38 (93.5% ± 3.5%) and CD95 (99.7% ± 0.1%; Supplementary Fig. S3). This approach to propagation yielded >10$^9$ γδ T cells from <10$^6$ total initiating cells (Fig. 1B), which represented a $4.9 \times 10^3 \pm 1.7 \times 10^3$ (mean ± SD; n = 4) fold increase. Thus, aAPCs with recombinant human cytokines supported the robust numeric expansion of polyclonal γδ T cells from small starting numbers of γδ T cells derived from PBMCs.

The addition of exogenous cytokines and presence of mIL15, CD86, and CD137L on clinical grade aAPCs were assessed for their ability to support the outgrowth of γδ T cells. Parental K562 cells were stably transfected with *Sleeping Beauty* (SB) transposons to introduce individual stimulatory molecules, cloned to achieve homogeneous expression (Supplementary Fig. S4), and then used to assess their impact on γδ T cell proliferation. Cocultures with exogenous IL2 and IL21 were initiated with paramagnetic bead purified γδ T cells and five sets of γ irradiated K562: (i) parental, (ii) mIL15$^+$, (iii) mIL15$^+$CD86$^+$, (iv) mIL15$^+$CD137L$^+$, and (v) mIL15$^+$CD86$^+$CD137L$^+$ (clone #4). γδ T cells cultured in parallel without APC demonstrated that soluble IL2 and IL21 sustained only limited numeric expansion of γδ T cells (Fig. 1C). Propagation improved upon addition of parental K562 cells, indicating that endogenous molecules on these cells can activate γδ T cells for proliferation. The expression of mIL15 with or without CD86 did not further improve the ability of γδ T cells to propagate compared with parental K562. In contrast, improved rates of propagation of γδ T cells were observed upon coculture with mIL15$^+$CD137L$^+$ and mIL15$^+$CD86$^+$CD137L$^+$ aAPCs. Thus, it appears that CD137L on aAPC clone#4 provides a dominant costimulatory proliferative signal for γδ T cells. In the absence of IL2 and IL21, the proliferation of γδ T cells ceased on aAPC clone#4, and together these cytokines exhibited an additive benefit to the rate of γδ T cell propagation (Fig. 1D). This validated our approach to combining aAPC clone #4 with cytokines to sustain the proliferation of polyclonal γδ T cells *ex vivo*, and demonstrated that CD137L on aAPCs, IL2, and IL21 were driving factors for proliferation of polyclonal γδ T cells to clinical scale.

### Ex vivo numeric expansion of neonatal γδ T cells on aAPCs in presence of IL2 and IL21

Allogeneic UCB is an important source of γδ T cells for adoptive transfer, because it contains younger cells and a more diverse TCRγδ repertoire relative to PBMCs, which
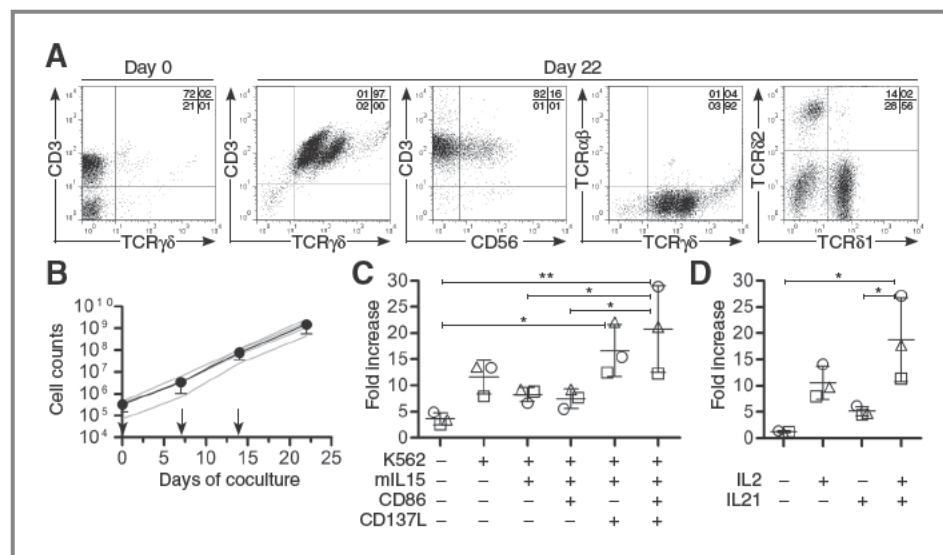
**Figure 1.** Sustained proliferation of polyclonal PBMC derived γδ T cells on γ irradiated aAPCs in the presence of soluble IL2 and IL21. A, frequency of γδ T cells before (day 0) and after (day 22) coculture on γ irradiated aAPCs, IL2, and IL21 where expression of CD3, CD56, TCRαβ, TCRγδ, TCRδ1, and TCRδ2 is shown at day 22 of coculture. One of 7 representative donors is shown. Quadrant frequencies (percentage) within flow plots are displayed in top right corners. B, inferred cell counts of polyclonal γδ T cells are displayed calculated on the basis of weekly yields and relative fold changes, where three arrows represent addition of aAPCs. Black line, mean ± SD ($n = 4$) pooled from 2 independent experiments and each gray line is an individual donor. C, fold increase over 9 days of γδ T cells cocultured with IL2 and IL21 along with aAPC expressing membrane bound IL15 (mIL15), CD86, and/or CD137L. Data, mean ± SD ($n = 3$) pooled from two independent experiments and each shape represents an individual donor. Two way ANOVA with Bonferroni posttests was used for statistical analysis. *, $P < 0.05$; **, $P < 0.01$. D, fold increase over 9 days of γδ T cells cocultured with aAPCs (clone #4) in the presence of either soluble recombinant IL2 and/or IL21. Data are mean ± SD ($n = 3$) pooled from two independent experiments where each shape represents an individual donor. Two way ANOVA with Bonferroni post tests was used for statistical analysis. *, $P < 0.05$.

could increase the number of ligands targeted by the engrafted cells and result in long term engraftment in the recipient (31). However, the limited number of mononuclear cells within a banked UCB unit curtails the number of neonatal γδ T cells directly available for adoptive transfer. Thus, we evaluated whether aAPCs could sustain proliferation from small starting numbers of neonatal γδ T cells. Fluorescence activated cell sorting (FACS) was used to isolate $10^4$ UCB derived γδ T cells (∼0.01% of a typical UCB unit) which were cocultured on aAPC clone #4 with IL2 and IL21. After 35 days, there was a $10^7$ fold increase in cell number, as an average of $10^{11}$ UCB derived γδ T cells (range: $6 \times 10^9$ $3 \times 10^{11}$; $n = 5$) were propagated from the $10^4$ initiating γδ T cells (Supplementary Fig. S5A). Two additional stimulations were performed for γδ T cells derived from UCB compared with PBMCs highlighting their potential for proliferating to clinically appealing numbers. The propagated γδ T cell populations exhibited uniform coexpression of CD3 and TCRγδ and lacked TCRαβ+ T cells or presence of CD3neg CD56+ NK cells (Supplementary Fig. S5B S5D). Collectively, these data demonstrate that aAPC clone #4 with IL2 and IL21 could sustain the *ex vivo* proliferation of γδ T cells from a small starting population of neonatal UCB.

### *Ex vivo* activated and propagated γδ T cells express polyclonal and defined TCRγδ repertoire

Upon establishing that γδ T cells could numerically expand on aAPCs and selected cytokines, we sought to determine the TCR repertoire of the propagated cells. Before numeric expansion, resting γδ T cell repertoire followed TCRδ2>TCRδ1neg TCRδ2neg>TCRδ1 by flow cytometry (Supplementary Fig. S6). However, the γδ T cell repertoire followed TCRδ1>TCRδ1neg TCRδ2neg>TCRδ2 following expansion, suggesting that there was a proliferative advantage for Vδ1 cells within polyclonal γδ T cell cultures. To look more in depth at TCRγδ diversity in aAPC expanded γδ T cells, we adapted a nonenzymatic digital multiplex assay used to quantify the TCR diversity in γδ T cells expressing a CD19 specific chimeric antigen receptor (CAR; ref. 27) termed DTEA. After expansion (day 22), 4 of 8 Vδ alleles (*TRDV1*, *TRDV2 2*, *TRDV3*, and *TRDV8*) were detected in PBMC derived γδ T cells (Fig. 2A) and were coexpressed with Vγ alleles *TRGV2*, *TRGV7*, *TRGV8* (two probes), *TRGV9*A1*, *TRGV10*A1*, and *TRGV11*02* (Fig. 2B). Similarly, a polyclonal assembly of Vδ and Vγ chains was observed in γδ T cells from UCB following expansion (days 34 35), albeit with reduced abundance of *TRDV2 2*, more *TRGV2*, and presence of *TRGV3F*, *TRDV5*, and *TRDV7* cells not seen from PBMCs (Fig. 2C and D). Similar patterns of Vδ and Vγ mRNA usage were detected in PBMCs and UCB before and after expansion (Supplementary Fig. S7) although overall mRNA counts were fewer in the resting cells (day 0) relative to the activated γδ T cells. Thus, aAPC expanded γδ T cells maintain a polyclonal TCR repertoire from both PBMCs and UCB.

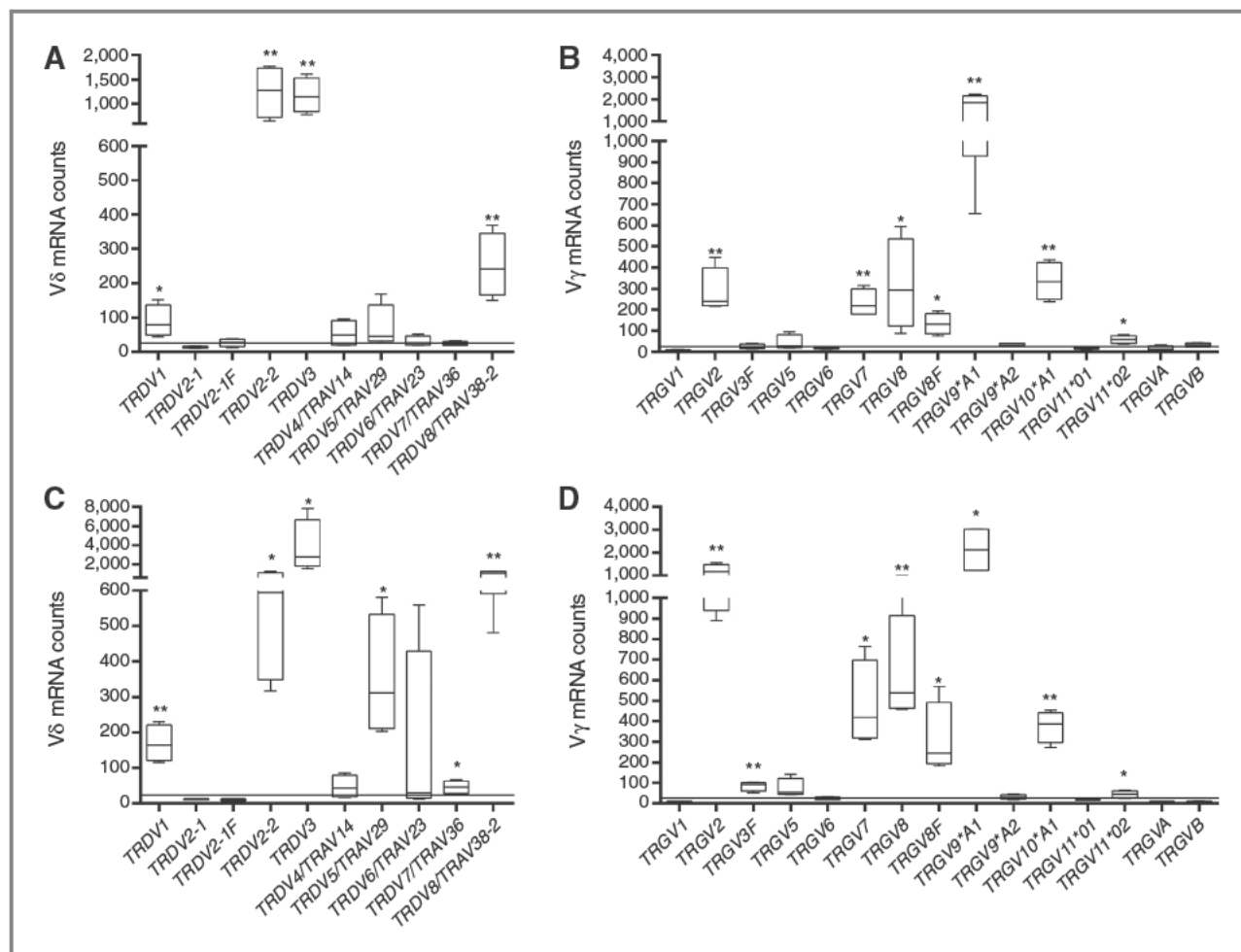We sought to validate these mRNA data by sorting polyclonal populations with TCRδ specific antibodies and

**Figure 2.** Abundance of Vδ and Vγ mRNA species in γδ T cells propagated and activated *ex vivo*. Quantification of mRNA species coding for (A) Vδ and (B) Vγ alleles in PBMC derived γδ T cells by DTEA at day 22 of coculture on aAPCs/IL2/IL21. Quantification of mRNA species coding for (C) Vδ and (D) Vγ alleles in UCB derived γδ T cells by DTEA at day 34 to 35 of coculture on aAPCs/IL2/IL21. Box and whiskers plots display 25% and 75% percentiles where lines represent maximum, mean, and minimum from top to bottom ($n = 4$). Solid lines at bottom of graphs represent limit of detection (LOD) calculated from mean $\pm 2 \times$ SD of DTEA negative controls. Student paired one tailed *t* tests were performed for each allele relative to the sample LOD. *, $P < 0.05$; **, $P < 0.01$.

repeating DTEA on isolated cultures. There are only two TCRδ specific mAbs commercially available and they identified three discrete Vδ populations (Vδ1: TCRδ1+TCRδ2neg, Vδ2: TCRδ1negTCRδ2+, and Vδ1negVδ2neg: TCRδ1negTCRδ2neg) within aAPC expanded γδ T cells from PBMCs (Fig. 1A) and UCB (Supplementary Fig. S8) with abundance following Vδ1>Vδ1negVδ2neg>Vδ2. FACS isolated subsets from PBMC derived γδ T cell pools were propagated with clone #4 as discrete populations and maintained their identity as assessed by expression of TCRδ isotypes (Fig. 3A). Each of the separated subsets could be identified by a pan specific TCRγδ antibody confirming that these cells were indeed γδ T cells (Fig. 3B). Furthermore, each population could be differentiated based on pan TCRγδ antibody mean fluorescence intensity (MFI) where Vδ2, Vδ1negVδ2neg, and Vδ1 T cells corresponded to the TCRγδlow (43 ± 9; mean ± SD; $n = 4$), TCRγδintermediate (168 ± 40), and TCRγδhi (236 ± 56) groupings, respectively. No differences in proliferation kinetics on

aAPCs were observed between isolated Vδ sorted subsets (Fig. 3C) indicating that the observed inversion of Vδ1 and Vδ2 frequencies in polyclonal cultures before versus after expansion was not due to a proliferative defect in one of the subsets. DTEA demonstrated that isolated and propagated Vδ1, Vδ2, and Vδ1negVδ2neg subpopulations were homogeneous populations as they predominantly expressed TRDV1, TRDV2 2, and TRDV3 mRNA species at 261 ± 35, 3,910 ± 611, and 5,559 ± 1119 absolute counts, respectively (Fig. 3D). Therefore, there were fewer TRDV1 mRNA species expressed by Vδ1 cells relative to the TRDV2 2 expressed by Vδ2 cells and TRDV3 expressed by Vδ1negVδ2neg cells. Moreover, these data indicated that the relatively low counts observed for TRDV1 in polyclonal populations with a preponderance of TCRδ1+ cells was not a defect in DTEA detection but rather a product of fewer total mRNA transcripts relative to other Vδ species. Given the wide range of mRNA
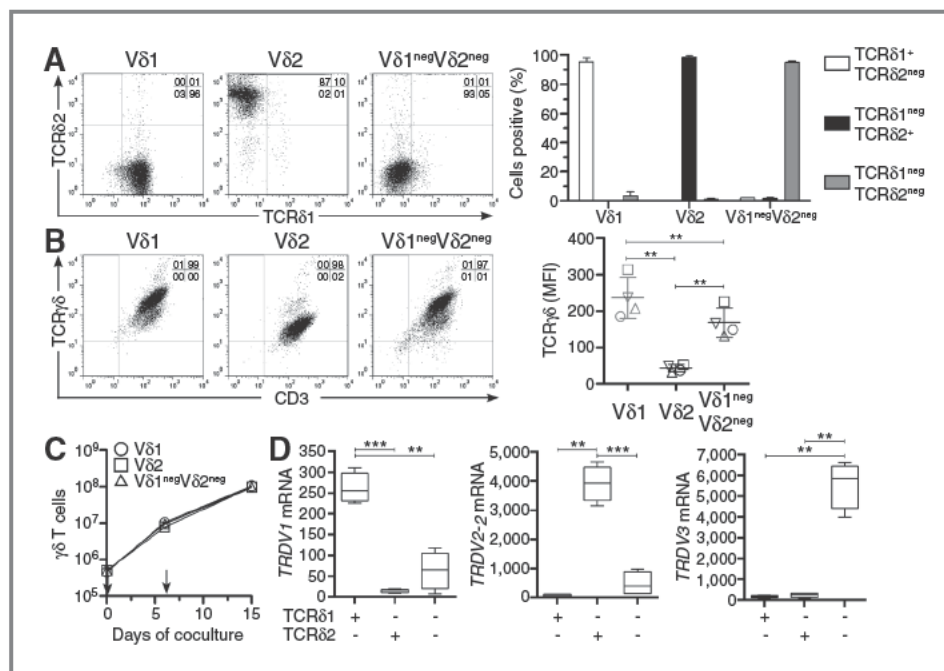
**Figure 3.** Sustained proliferation of PBMC derived Vδ T cell subsets expanded on γ irradiated aAPCs/IL2/IL21. After two 7 day stimulations with aAPCs (clone #4) and IL2/IL21, the bulk population of γδ T cells was separated into Vδ1, Vδ2, and Vδ1$^{neg}$Vδ2$^{neg}$ subsets by FACS based on staining of T cells defined as TCRδ1$^+$TCRδ2$^{neg}$, TCRδ1$^{neg}$TCRδ2$^+$, and TCRδ1$^{neg}$TCRδ2$^{neg}$, respectively. A, expression of TCRδ1 and TCRδ2 chains on Vδ1, Vδ2, and Vδ1$^{neg}$Vδ2$^{neg}$ subsets of γδ T cells (from left to right) after 15 days of numeric expansion on aAPCs and cytokines as isolated groups. One of 4 representative donors is shown pooled from two independent experiments. Quadrant frequencies (percentage) within flow plots are displayed in top right corners. Frequency of TCRδ1$^+$TCRδ2$^{neg}$ (open bars), TCRδ1$^{neg}$TCRδ2$^+$ (black bars), and TCRδ1$^{neg}$TCRδ2$^{neg}$ (gray bars) cell surface protein expression in subsets of γδ T cells after 15 days numeric expansion on aAPCs and cytokines as isolated groups. Data are mean ± SD ($n = 4$) pooled from two independent experiments. B, flow cytometry plots of CD3 and TCRγδ expression in Vδ1, Vδ2, and Vδ1$^{neg}$Vδ2$^{neg}$ subsets (from left to right). Mean fluorescence intensity (MFI) of TCRγδ staining in Vδ1, Vδ2, and Vδ1$^{neg}$Vδ2$^{neg}$ T cell subsets where each shape represents a different donor and data are mean ± SD ($n = 4$) pooled from two independent experiments. C, proliferation of each isolated Vδ subset stimulated twice with aAPC clone #4 (arrows) in presence of cytokines and total cell counts are displayed. Data are mean ± SD ($n = 4$) pooled from 2 independent experiments. D, DTEA was used to identify and measure abundance of mRNA species coding for TRDV1, TRDV2 2, and TRDV3 (from left to right) in γδ T cell subpopulations after 15 days of proliferation on aAPCs and cytokines as separated subsets. Box and whiskers plots display 25% and 75% percentiles where lines represent maximum, mean, and minimum from top to bottom ($n = 4$). Student paired, two tailed $t$ tests were undertaken for statistical analyses between groups. **, $P < 0.01$; ***, $P < 0.001$.

transcript quantities for each allele, DTEA was not useful for calculation of relative frequencies of Vδ subsets in polyclonal populations but rather was indicative of presence or absence of a particular γδ T cell subset. Expression of other Vδ2 alleles (TRDV2 1 and TRDV2 1F) was absent from polyclonal γδ T cells (Fig. 2A) and each of the sorted subsets (data not shown). Small amounts of TRDV4, TRDV5, TRDV6, and TRDV7 mRNA species were detected in the three subsets of T cells sorted for Vδ expression (Supplementary Fig. S9). TRDV8 mRNA was exclusively present in sorted Vδ1$^{neg}$Vδ2$^{neg}$ cells and these T cells are likely the main contributors of TRDV8 in bulk γδ T cells. The same Vγ mRNA present in polyclonal cultures was detected in Vδ sorted cultures (Supplementary Fig. S10). Furthermore, Vδ1 and Vδ1$^{neg}$Vδ2$^{neg}$ were not different ($P = 0.419$; two way ANOVA) but Vδ2 was different to both Vδ1 ($P < 0.0001$) and Vδ1$^{neg}$Vδ2$^{neg}$ ($P < 0.0001$) in Vγ usage. Collectively, these results confirmed DTEA from unsorted cultures and strongly supported the polyclonal TCRγδ expression on γδ T cells activated to proliferate by aAPCs and cytokines.

## IFNγ produced in response to tumors is dependent on TCRγδ

A multiplex analysis of cytokines and chemokines was performed to determine whether aAPC propagated γδ T cells might foster a proinflammatory response in a tumor microenvironment (Fig. 4A). The Th1 associated cytokines IFNγ and TNFα were secreted in abundance by γδ T cells upon exposure to leukocyte activated cocktail (LAC; PMA and ionomycin for nonspecific mitogenic stimulation), in addition to small amounts of IL2 and IL12 p70. In contrast, no significant production of the Th2 associated cytokines IL4, IL5, and IL13 was observed from LAC treated γδ T cells, but there was a small increase in IL10 production over baseline. Similarly, Th17 associated cytokines IL1RA, IL6, and IL17 were secreted at low levels by LAC treated γδ T cells. The chemokines CCL3, CCL4, CCL5, and CXCL8 were detected in abundance. Minor contributions of non γδ T cells in the culture that could have been activated by LAC to secrete cytokines could not be ruled out, but given that the cells tested were 97.9% ± 0.6% CD3$^+$TCRγδ$^+$ these data indicate that it was activation of γδ T cells that led to a largely proinflammatory response. IFNγ was the most responsive
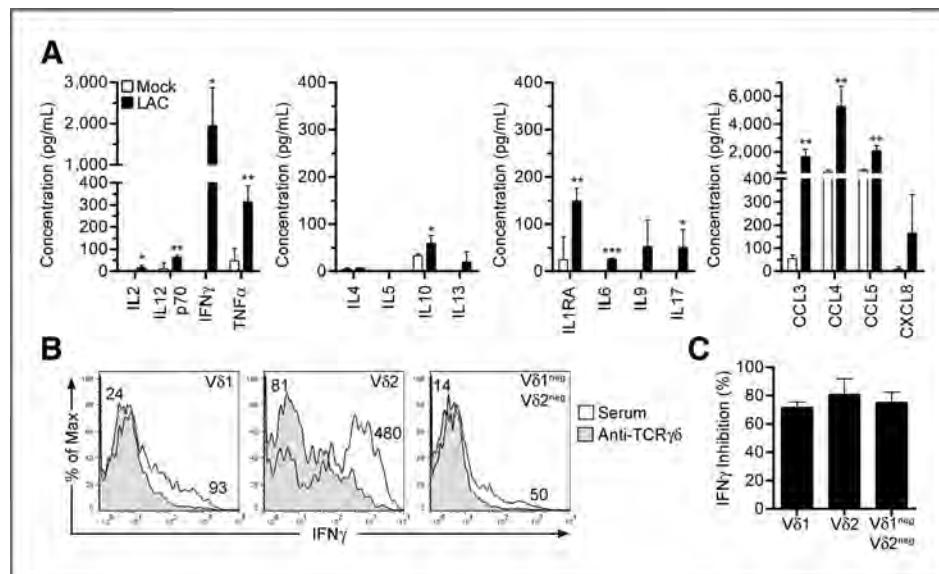
**Figure 4.** Dependence on TCRγδ for IFNγ secretion in response to tumor cells. At day 22 of coculture on γ irradiated aAPCs (clone #4) with IL2 and IL21, T cells were incubated with media (mock) or leukocyte activation cocktail (LAC; PMA/Ionomycin) for 6 hours at 37°C. Tissue culture supernatants were interrogated using 27 Plex Luminex array to detect presence of (A) Th1, Th2, and Th17 cytokines and selected chemokines (from left to right). Data are mean ± SD pooled from 4 donors in two independent experiments where each donor had triplicate experimental wells pooled before multiplex analysis. Student one tailed $t$ test performed for statistical analysis between mock and LAC groups. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$. B, polyclonal γδ T cells were incubated for 1 hour before and during 6 hour tumor cell coculture with normal mouse serum or neutralizing TCRγδ antibody (clone IM). Cells were stained for TCRδ1, TCRδ2, CD3, and IFNγ to gate T cell subsets and assess IFNγ production. Comparisons of histograms detailing Vδ1, Vδ2, and Vδ1$^{neg}$Vδ2$^{neg}$ gates (from left to right) cocultured with CAOV3 ovarian cancer cells and treated with serum (open) or TCRγδ (shaded). Numbers next to histograms are MFI. Flow plots are representative of 1 of 3 peripheral blood donors cocultured with CAOV3 cells in 2 independent experiments. C, percent inhibition of IFNγ secretion in response to CAOV3 cells was calculated for each Vδ T cell subset based on the following equation: Inhibition (%) = 100 − 100 × [(MFI$_{TUMOR + T CELL}$ − MFI$_{T CELL ONLY}$)$_{TCRγδ}$/(MFI$_{TUMOR + T CELL}$ − MFI$_{T CELL ONLY}$)$_{Serum}$]. Data are mean ± SD ($n = 3$) pooled from two independent experiments.

of all the assessed cytokines and was chosen to measure responses of Vδ subsets to tumor cells (Fig. 4B). Coculture of polyclonal aAPC propagated/activated γ δ T cells with can cer cells resulted in a hierarchy of IFNγ production follow ing Vδ2>Vδ1>Vδ1$^{neg}$Vδ2$^{neg}$ as shown by MFI of 855 ± 475, 242 ± 178, and 194 ± 182 (mean ± SD; $n = 4$), respectively. IFNγ production by Vδ1, Vδ2, and Vδ1$^{neg}$Vδ2$^{neg}$ subsets was inhibited by pan TCRγδ antibody when added to γδ T cell/tumor cocultures indicating that response to the tumor in each subset was dependent upon activation through TCRγδ (Fig. 4C). This observation supported the premise that a single cancer cell could be targeted by discrete γδ TCRs. Thus, a multivalent proinflammatory response to the tumor cell was achieved by polyclonal γδ T cells.

### Polyclonal γδ T cells lyse a broad range of tumor cells through combination of DNAM1, NKG2D, and TCRγδ

After establishing that propagated γδ T cells could be activated to produce proinflammatory cytokines, we exam ined their ability to specifically lyse a panel of tumor cell lines. Polyclonal γδ T cells demonstrated a range of cytolysis against solid and hematologic cancer cell lines without a clear preference towards a particular tumor histology or grade (Fig. 5 and Supplementary Fig. S11). We previously established that B cell acute lymphoblastic leukemia (ALL) cell line NALM 6 was largely resistant to lysis by γδ T cells,

which required a CD19 specific CAR to acquire significant killing capability (27). In this study, it was also observed that autologous and allogeneic normal B cells were spared from cytolysis (Fig. 5A), and that B ALL cell line cALL 2 and murine T cell lymphoma cell line EL4 were lysed poorly by polyclonal γδ T cells, which indicated that some cells were resistant and/or not recognized by polyclonal γδ T cells. In contrast, T ALL cell line Jurkat and B ALL cell lines RCH ACV were both killed efficiently by polyclonal γδ T cells (Fig. 5B), indicating that γδ T cells could be used to target some B cell and T cell malignancies. Kasumi 3 is a CD33$^+$ CD34$^+$ undifferentiated leukemia cell line that was lysed at intermediate levels by γδ T cells. Chronic myelogenous leukemia (CML) cell line K562 and K562 derived clone#4 aAPCs were killed by polyclonal γδ T cells, which corrob orated the notion that these cells could serve as a prolifer ative substrate. Pancreatic cancer cell lines BxPc 3, Mia PaCa 2, and Su8686, were lysed by γδ T cells, as was the colon carcinoma cell line HCT 116 (Fig. 5C). Ovarian cell lines were killed by polyclonal γδ T cells in the following order of decreasing sensitivity: CAOV3 > EFO21 > UPN251 > IGROV1 > OC314 > Hey > A2780 > OVCAR3 > OAW42 > EFO27. Each of the separated Vδ subsets lysed hematologic (Jurkat and K562) and solid (OC314 and CAOV3) tumor cell lines, which showed that polyclonal γδ T cells could direct a multivalent response against common targets (Sup plementary Fig. S12). The strength of cytolysis followed the
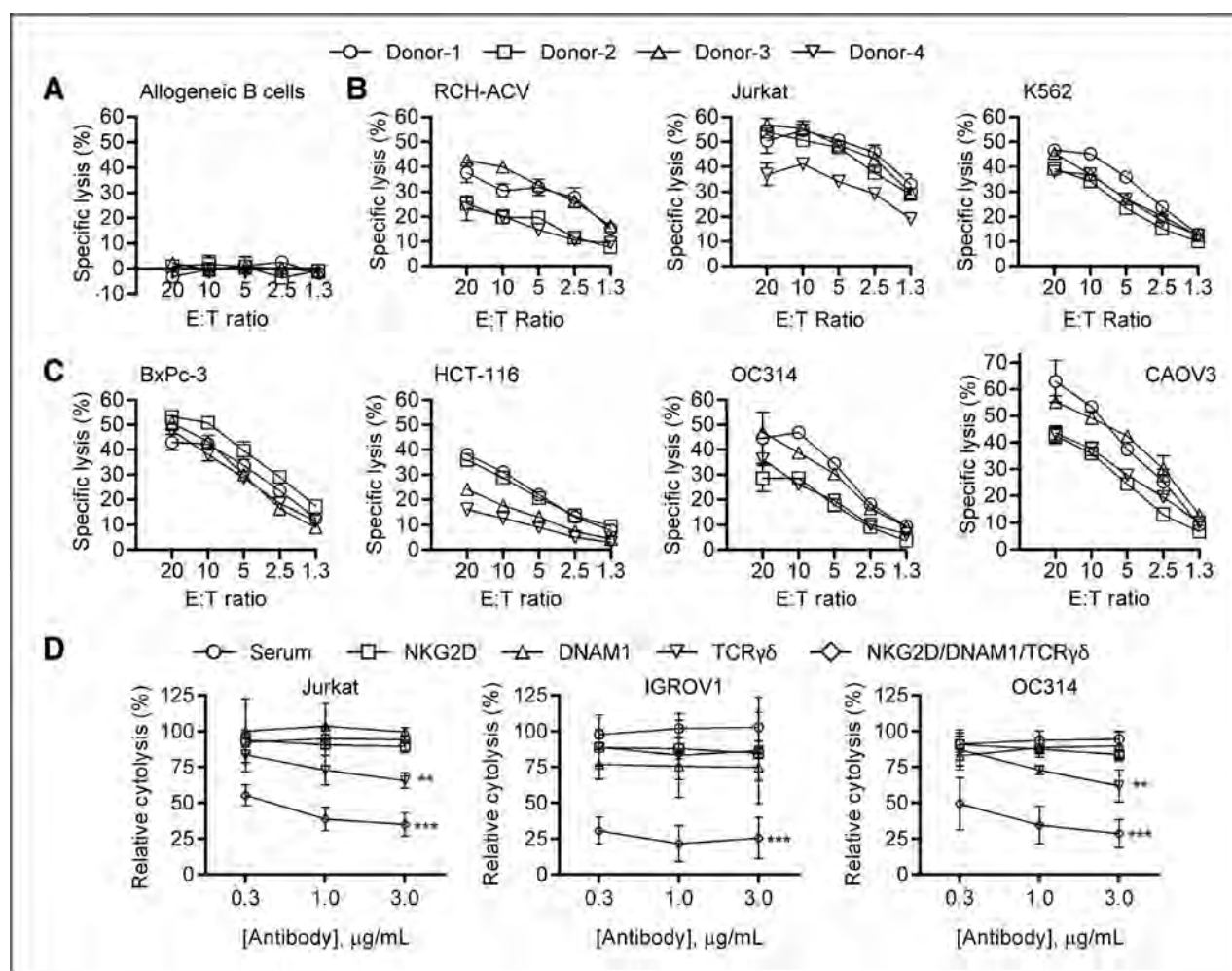
**Figure 5.** Specific lysis of tumor cell panel by polyclonal $\gamma\delta$ T cells. A  C, standard 4  hour CRA was performed with increasing effector (polyclonal $\gamma\delta$ T cells; each shape represents a different donor) to target (E:T) ratios against (A) healthy B cells from an allogeneic donor (one of four representative donors); B, hematologic tumor cell lines derived from B ALL: RCH ACV, T ALL: Jurkat, and CML: K562; C, solid tumor cell lines derived from pancreatic cancer: BxPc 3, colon cancer: HCT 116, and ovarian cancer: OC314 and CAOV3. Data are mean $\pm$ SD ($n = 3$ wells/assay) from two independent experiments. D, neutralizing antibodies to NKG2D (squares), DNAM1 (triangles), TCR$\gamma\delta$ (inverted triangles), or a pool (diamonds) of all three antibodies were used to block killing of Jurkat (left), IGROV1 (middle), or OC314 (right) tumor targets antibodies at 0.3, 1, and 3 $\mu$g/mL and an E:T ratio of 12:1 in standard 4 hour CRA. Normal mouse serum (circles) served as control for addition of antibody and wells without antibody were used for normalization purposes. Specific lysis was normalized to wells without antibody to yield relative cytolysis as defined by: Relative cytolysis (%) = (Specific Lysis)$_{\text{With Antibody}}$/(Specific Lysis)$_{\text{Without Antibody}} \times$ 100. Data are mean $\pm$ SD ($n = 4$ donors) from triplicates pooled and normalized from two independent experiments. Repeated measures two way ANOVA was used for statistical analysis between antibody treatments. $^{**}$, $P < 0.01$; $^{***}$, $P < 0.001$.

hierarchy of TCR usage (V$\delta$2>V$\delta$1$^{\text{neg}}$V$\delta$2$^{\text{neg}}$>V$\delta$1) that was consistent with the premise that a propensity to be triggered for effector function would increase with T cell differentiation (Supplementary Fig. S13). Lysis by poly clonal populations was apparently not due to one specific V$\delta$ subtype but rather from contributions of multiple $\gamma\delta$ T cell subsets, because it was observed that (i) a number of tumor cell lines were equivalently killed by polyclonal $\gamma\delta$ T cells containing different frequencies of V$\delta$1, V$\delta$2, and V$\delta$1$^{\text{neg}}$V$\delta$2$^{\text{neg}}$ cells and (ii) a polyclonal population was not identified with dominant cytolysis. We also sought to determine which surface molecules were responsible for cytolysis by blocking immunoreceptors

with antibodies (Fig. 5D). Our experimental approach also took into account that $\gamma\delta$ T cells coexpress DNAM1 (97.7% $\pm$ 0.9%; mean $\pm$ SD; $n = 4$) and NKG2D (40.1% $\pm$ 16.5%) which can activate both T cells and NK cells for killing (32, 33). Addition of individual antibodies did not reduce lysis, except for TCR$\gamma\delta$ in 2 of 3 cell lines tested. In contrast, a pool of antibodies binding NKG2D, DNAM1, TCR$\gamma\delta$ resulted in significant inhibition, in a dose depen dent manner, of $\gamma\delta$ T cell  mediated cytolysis against all 3 targets. Collectively, these data established that *ex vivo* propagated $\gamma\delta$ T cells have broad antitumor capabilities likely mediated by activation though DNAM1, NKG2D, and TCR$\gamma\delta$.
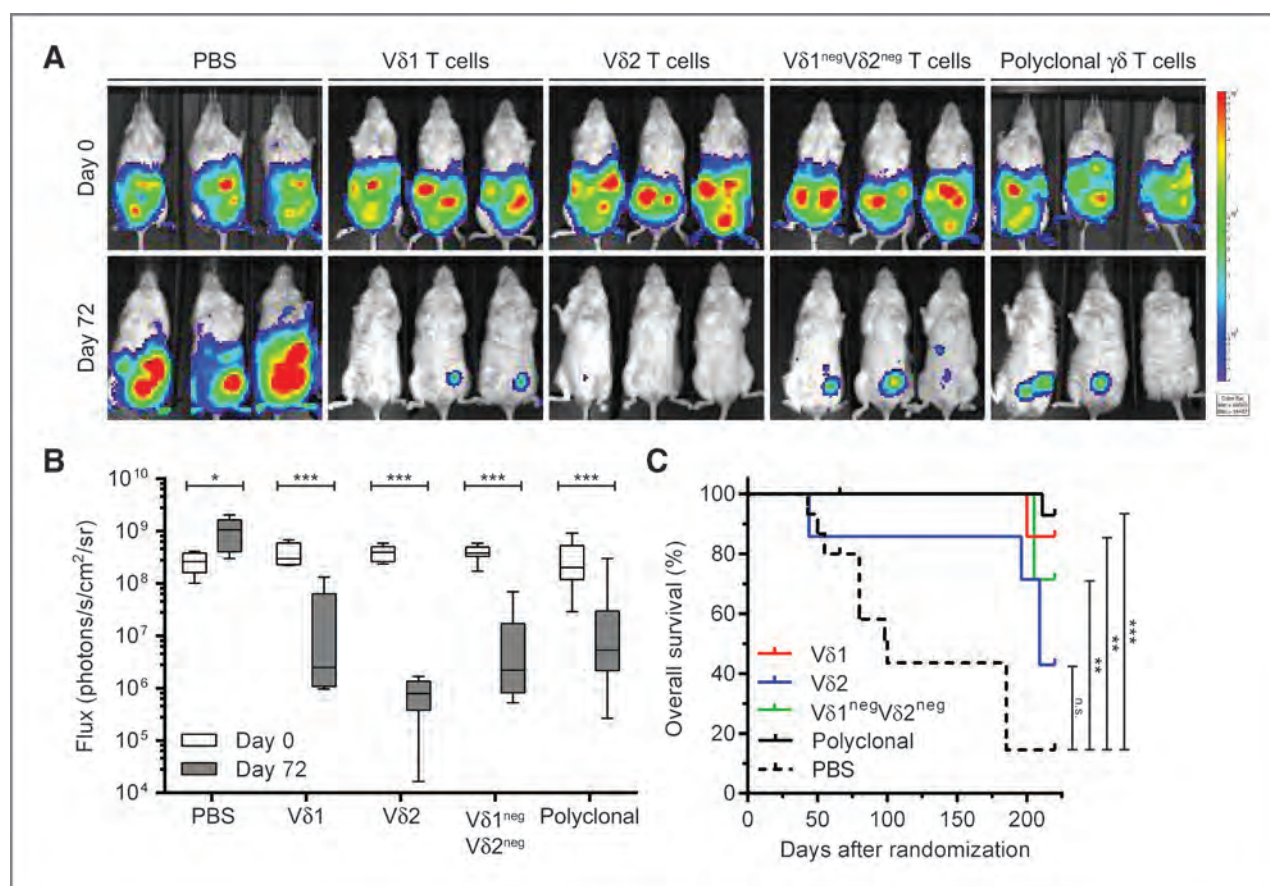
**Figure 6.** *In vivo* clearance of ovarian cancer upon adoptive transfer of polyclonal γδ T cells and γδ T cell subsets propagated/activated on aAPCs with IL2 and IL21. CAOV3 *effLuc* mKate tumor cells were injected into NSG mice at day 8 and engrafted until day 0 when treatment was started with either PBS (vehicle/mock) or γδ T cells. Four T cell doses were administered in weekly escalating doses. A, BLI images at day 0 (top) or day 72 (bottom) in PBS, Vδ1, Vδ2, Vδ1$^{neg}$Vδ2$^{neg}$, and polyclonal γδ T cell treatment groups. Images are representative of 6 to 14 mice from two independent experiments. B, BLI measurements of mice at day 0 (white) and day 72 (gray) pooled from two independent experiments. Box and whiskers plots display 25% and 75% percentiles where lines represent maximum, mean, and minimum from top to bottom ($n = 6\ 14$). Student paired, two tailed *t* tests were used for statistical analysis between time points. C, overall survival of mice treated with PBS (dashed), polyclonal (black), Vδ1 (red), Vδ2 (blue), or Vδ1$^{neg}$Vδ2$^{neg}$ (green) γδ T cells. Log rank (Mantel Cox) test was used to calculate *P* values. *, $P < 0.05$; **, $P < 0.01$; and ***, $P \leq 0.001$.

## Established ovarian cancer xenografts are eliminated by adoptive transfer of γδ T cells

To test whether polyclonal γδ T cells were effective in targeting and killing tumors *in vivo*, we created a xenograft model for ovarian cancer in immunocompromised mice. NSG mice were injected intraperitoneally with CAOV3 *effLuc* mKate ovarian cancer cells and then randomized into five treatment groups. After 8 days of tumor engraftment, either PBS (vehicle/mock), Vδ1, Vδ2, Vδ1$^{neg}$Vδ2$^{neg}$, or polyclonal γδ T cells were administered in escalating doses (Fig. 6). Tumor burden and biodistribution were serially measured by noninvasive BLI. Established tumors continued to grow in vehicle treated mice, but tumor bio burden was significantly reduced ($P \leq 0.001$) in mice receiving γδ T cell treatments at day 72, relative to their initial tumor burden (Fig. 6A and B). Adoptive transfer of polyclonal γδ T cells, Vδ1, and Vδ1$^{neg}$Vδ2$^{neg}$ T cells signif icantly ($P \leq 0.01$), and Vδ2 almost significantly ($P = 0.055$), increased long term survival compared with mock treated

mice. This corresponded to overall survival following poly clonal>Vδ1>Vδ1$^{neg}$Vδ2$^{neg}$>Vδ2 (Fig. 6C). This is the first time that three Vδ subsets have been compared for their ability to target tumor *in vivo* and is the first display of *in vivo* antitumor activity by Vδ1$^{neg}$Vδ2$^{neg}$ cells. In sum, activated and propagated γδ T cells were effective in treating cancer *in vivo* and thus represent an attractive approach to adoptive immunotherapy.

## Discussion

This study establishes our aAPC clone #4 as a cellular platform for the sustained proliferation of multiple γδ T cell populations that demonstrate extensive reactivity against hematologic and solid malignancies. T cells expressing defined Vδ TCRs have been associated with clinical responses against cancer. For example, the Vδ1 subset correlated with complete responses observed in patients with ALL and acute myelogenous leukemia (AML) after αβ

T cell depleted haploidentical HSCT (15 17). Vδ1 cells were also shown to kill glioblastoma independent of cyto megalovirus (CMV) status (34). However, Vδ1 cells have not been directly administered. Our data establish that such cells could mediate antitumor immunity and supports the adoptive transfer Vδ1 T cells for cancer therapy. In contrast to Vδ1 and Vδ1$^{neg}$Vδ2$^{neg}$ cells, T cells expressing Vδ2 TCR have been directly infused and elicited responses against solid and hematologic tumors (9, 35). Little is known about Vδ1$^{neg}$Vδ2$^{neg}$ T cells, but these lymphocytes have displayed recognition of the nonclassical MHC molecule CD1d with corresponding NKT like functions and have also been cor related with immunity to HIV and CMV (36 39). Our results are the first to directly show that Vδ1$^{neg}$Vδ2$^{neg}$ cells exhibit antitumor activities, and given their propensity to engage both viruses and cancer the add back of this subset could especially benefit immunocompromised cancer patients. Because aAPCs with IL2 and IL21 can propagate polyclonal γδ T cells, mAbs can now be raised against Vδ3, Vδ5, Vδ7, and Vδ8 isotypes to help elucidate their potential roles in clearance of pathogens and cancer. In aggregate, our data support the adoptive transfer of γδ T cells that maintain expression of multiple Vδ TCR types as investigational treatment for cancer.

The molecules on aAPCs that activate γδ T cells for numeric expansion are not well known. K562 derived aAPCs express endogenous MHC class I chain related pro tein A and B (MICA/B) which are ligands for both Vδ1 and NKG2D (6, 40). Indeed, NKG2D was observed on poly clonal γδ T cells that also predominantly expressed Vδ1 TCR (Fig. 1A). Polyclonal γδ T cells also demonstrate expression for activating receptors typically found on NK cells (NKp30, NKp44, and NKp46; collectively expressed at 26% ± 7%), and future studies will examine their contribution to γδ T cell effector function. Some malignant cells were recognized poorly by γδ T cells, for e.g., EL4, EFO27, OAW42, cALL 2, and NALM 6, which provides an opportunity to further interrogate the mechanism by which γδ T cells recognize and kill tumor cells. Given that inhibition of cytolysis was maximized by neutralizing DNAM1, NKG2D, and TCRγδ receptors simultaneously, it may be that sensitivity of a tumor cell resides on the expression of ligand combinations that can bind these receptors. Two ligands recognized by Vδ2 TCR are surface mitochondrial $F_1$ ATPase and phos pho antigens, both of which are found in K562 cells (41, 42). Enhanced responses of T cells expressing Vγ9Vδ2 were observed when K562 cells were treated with aminobispho sphonates (41) and a similar strategy could be employed upon coculture with aAPC clone #4 to increase the abun dance of T cells bearing Vδ2 TCR (18). Future studies will evaluate additional TCRγδ ligands that naturally occur in these aAPCs.

We enforced expression of costimulatory molecules to ascertain and improve the capability of K562 derived aAPCs to propagate γδ T cells expressing a diversity of TCR. Indeed, CD137L was the dominant costimulatory proliferative sig nal on aAPCs for expansion of γδ T cells with broad tumor reactivity (Fig. 1C), and its receptor, CD137, has been used to enrich tumor reactive αβ T cells following antigen expo sure and presumably TCR stimulation (43 45). CD137 was not expressed on resting γδ T cells before expansion, sug gesting that the importance of CD137L costimulation by aAPCs followed TCR stimulation by the aAPCs and expres sion of CD137 on the γδ T cell surface. CD27$^+$ and CD27$^{neg}$ γδ T cells have been shown to produce IFNγ and IL17 (46), respectively; therefore, CD27 could be used as a marker for isolating γδ T cells with a preferred cytokine output. ICOS ligand in absence of CD86 was shown to polarize CD4$^+$ αβ T cells to produce IL17 instead of IFNγ (47), and current studies are investigating whether combinations of costimu latory molecules can selectively propagate cytokine produc ing subpopulations of γδ T cells. Thus, the aAPC coculture system in the context of desired cytokines provides a clin ically relevant methodology to tailor the type of therapeutic γδ T cell produced for adoptive immunotherapy.

Our data have implications for the design and inter pretation of clinical trials. Expression of IL15 was impor tant for the maintenance of transferred γδ T cells in vivo (48), supporting the use of IL15 on aAPCs, and future studies could inform on other molecules that could be introduced to maximize the cell therapy product. Correl ative studies are enhanced by our observation that TCRγδ mAb can be used to readily distinguish the three (Vδ1, Vδ2, and Vδ1$^{neg}$Vδ2$^{neg}$) T cell subsets based on MFI of TCRγδ expression (Fig. 3B). Given that γδ T cells are not thought to recognize ligands in the context of MHC (17), there is potential to infuse allogeneic, including third party, γδ T cells in lymphodepleted hosts to achieve an antitumor effect while mitigating the risk of graft versus host disease. Restoration of lymphopoiesis may result in graft rejection, but a therapeutic window could be estab lished whereby tumors are directly killed by infused γδ T cells, which may result in desired bystander effects as conserved or neoantigens are presented to other lympho cytes. Indeed, γδ T cells have been shown to lyse cancer cells, cross present tumor specific antigens to αβ T cells, and license them to kill tumors (49, 50). The aAPC clone #4 has been produced as a master cell bank in compliance with current good manufacturing practice and provides a clear path to generating clinical grade γδ T cells for human application. Human trials can now, for the first time, test the efficacy of adoptive transfer of T cells with polyclonal TCRγδ repertoire for treatment of solid and hematologic tumors.

## Authors' Contributions

**Conception and design:** D.C. Deniger, S. Maiti, R.E. Champlin, L.J.N. Cooper
**Development of methodology:** D.C. Deniger, S. Maiti, M.H. Huls, D.A. Lee

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# Clinical applications of gamma delta T cells with multivalent immunity

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γδ T cells hold promise for adoptive immunotherapy because of their reactivity to bacteria, viruses, and tumors. However, these cells represent a small fraction (1–5%) of the peripheral T-cell pool and require activation and propagation to achieve clinical benefit. Aminobisphosphonates specifically expand the Vγ9Vδ2 subset of γδ T cells and have been used in clinical trials of cancer where objective responses were detected. The Vγ9Vδ2 T cell receptor (TCR) heterodimer binds multiple ligands and results in a multivalent attack by a monoclonal T cell population. Alternatively, populations of γδ T cells with oligoclonal or polyclonal TCR repertoire could be infused for broad-range specificity. However, this goal has been restricted by a lack of applicable expansion protocols for non-Vγ9Vδ2 cells. Recent advances using immobilized antigens, agonistic monoclonal antibodies (mAbs), tumor-derived artificial antigen presenting cells (aAPC), or combinations of activating mAbs and aAPC have been successful in expanding gamma delta T cells with oligoclonal or polyclonal TCR repertoires. Immobilized major histocompatibility complex Class-I chain-related A was a stimulus for γδ T cells expressing TCRδ1 isotypes, and plate-bound activating antibodies have expanded Vδ1 and Vδ2 cells ex vivo. Clinically sufficient quantities of TCRδ1, TCRδ2, and TCRδ1$^{neg}$TCRδ2$^{neg}$ have been produced following co-culture on aAPC, and these subsets displayed differences in memory phenotype and reactivity to tumors in vitro and in vivo. Gamma delta T cells are also amenable to genetic modification as evidenced by introduction of αβ TCRs, chimeric antigen receptors, and drug-resistance genes. This represents a promising future for the clinical application of oligoclonal or polyclonal γδ T cells in autologous and allogeneic settings that builds on current trials testing the safety and efficacy of Vγ9Vδ2 T cells.

**Keywords: cancer, immunotherapy, γδ T cells, adoptive T-cell therapy, T-cell receptor, allogeneic transplantation, chimeric antigen receptors, artificial APC**

## INTRODUCTION

γδ T cells possess a combination of innate and adaptive immune cell qualities rendering them attractive for immunotherapy (1–3). They can produce inflammatory cytokines, directly lyse infected or malignant cells, and establish a memory response to attack pathogens upon re-exposure. γδ T cells are defined by expression of γ and δ heterodimer of T cell receptor (TCR) chains (TCRγ/TCRδ) that directs intracellular signaling through associated CD3 complexes (4). The γδ T-cell lineage (1–5% of circulating T cells) can be contrasted to the more prevalent αβ T cell lineage (~90%) in peripheral blood, which expresses TCRα/TCRβ

heterodimers and also signals through associated CD3 complexes (5, 6). CD4 and CD8 co-receptors on αβ T cells assist binding of TCRαβ chains to the major histocompatibility complex (MHC) presenting processed peptides (7–9). In contrast, TCRγδ directly binds to an antigen's superstructure independent of the MHC/peptide complexes and, as a result, CD4 and CD8 are uncommon on γδ T cells (10, 11). Given that antigen recognition is achieved outside of MHC/peptide-restriction, γδ T cells have predictable immune effector functions mediated through their TCR and have potential use as universal ("off-the-shelf") allogeneic T-cell therapies (12).

Functional responses by γδ T cells can be stratified by the variable (V) region of the TCRδ chain. In humans, the TCRδ locus (*TRD*) lies within the TCRα locus (*TRA*). Three unique Vδ alleles, *TRDV1*, *TRDV2*, and *TRDV3*, code for TCRδ1, TCRδ2, and TCRδ3, respectively. Additionally, shared Vδ and Vα variable regions exist in *TRDV4/TRAV14*, *TRDV5/TRAV29*, *TRDV6/TRAV23*, *TRDV7/TRAV36*, and *TRDV8/TRAV38-2* loci. Recombination of these shared V alleles with a *TRA* junction region (*TRAJ*) results in TCRα14, TCRα29, TCRα23, TCRα36,

**Abbreviations:** 2M3B1PP, 2-methyl-3-butenyl-1-pyrophosphate; AML, acute myeloid leukemia; BrHPP, bromohydrin pyrophosphate; CLL, chronic lymphocytic leukemia; CRC, colorectal cancer; EOC, epithelial ovarian cancer; FCL, follicle center lymphoma; GI-cancer, cancers from the gastrointestinal tract; HIV, human immunodeficiency virus; HRPC, hormone-refractory prostate cancer; IC, immunocytoma; MM, multiple myeloma; MZL, mantle zone lymphoma; N/D, not determined; NHL, T-cell non-Hodgkin lymphoma; NSCLC, non-small-cell lung cancer; RCC, renal cell carcinoma; TBI, total body irradiation; T-SPL, secondary plasma cell leukemia; Zol, zoledronic acid.

and TCRα38-2, respectively, but recombination of these shared V alleles with *TRD* junction (*TRDJ*) and diversity (*TRDD*) regions results in TCRδ4, TCRδ5, TCRδ6, TCRδ7, and TCRδ8, respectively (13). Expression of TCRγδ heterodimers on the T-cell surface in the thymus inhibits recombination of TCRβ-chain locus during the $CD4^{neg}CD8^{neg}$ stage thereby committing the T cell to the γδ T-cell lineage (14). This double negative status is typically maintained upon exit from the thymus, most likely because co-receptors are dispensable for functional TCRγδ binding to antigens (15). However, the thymus is not required to complete all γδ T-cell development, as many γδ T cells directly take up residence in peripheral tissues following exit from the bone marrow and exhibit immediate effector functions against pathogens (16). Thymus-independent "resident" γδ T cells can be found in the mucosa, tongue, vagina, intestine, lung, liver, and skin and can comprise up to 50% of the T-cell populations in intestinal epithelial lymphocytes (17, 18). In contrast, circulating γδ T cells can be found in the blood and lymphoid organs, and are dominated by γδ T cells preferentially expressing TCRδ2 isotype (commonly referred to as Vδ2 cells). Indeed, γδ T cells expressing the TCRδ1 isotype (commonly referred to as Vδ1 cells) are frequently found within tissues (19, 20). Vδ2 cells have preferred pairing with TCRγ9 (Vγ9Vδ2 cells), but broad γ-chain pairing is observed in Vδ1 cells and $Vδ1^{neg}Vδ2^{neg}$ cells, a generic grouping of all other non-Vδ1/Vδ2 T cells (12, 19). Therefore, γδ T cells are distributed across an array of anatomical locations with a range of TCRγδ variable region expression.

Human TCRγδ ligands are MHC/peptide complex-independent and are therefore conserved amongst unrelated individuals. Most of the known human ligands are specific for TCRδ1 or TCRδ2. TCRγ1/TCRδ1 (alternatively termed Vγ1Vδ1) heterodimers have specificity for MHC Class-I chain-related A (MICA) (21, 22), a molecule participating in evasion of immune surveillance following viral infection and expressed on tumor cells as it is involved in the cellular stress response (23). MICA is also one of the ligands for NKG2D, which is expressed on γδ T cells, αβ T cells, and natural killer (NK) cells (23, 24). Both Vγ1Vδ1 and Vγ2Vδ1 recognize non-polymorphic MHC molecule CD1c (25), and Vγ5Vδ1 is a receptor for α-galactosylceramide-CD1d complexes commonly described in the activation of natural killer T (NKT) cells which, like γδ T cells, have both innate and adaptive immune functions and recognize conserved ligands amongst unrelated individuals (26, 27). γδ T cells can have specificity for virus as cytomegalovirus (CMV)-reactive Vγ8Vδ1 cells have been isolated from umbilical cord blood from infected newborns (28). Vδ1 cells have also been associated with immunity to human immunodeficiency virus (HIV), but the precise HIV ligands for TCRδ1 have not been determined (29). Bacterial alkylamines and *Listeria monocytogenes* are recognized by Vδ2 cells when paired with Vγ2 (30–32). Vγ9Vδ2 cells are the most extensively studied sub-group of human γδ T cells and their ligands include phosphoantigens [isopentenyl pyrophosphate (IPP)], $F_1$-ATPase expressed on the cell surface, apolipoprotein A-I, and *Mycobacterium tuberculosis* (33–37). Moreover, Vγ9Vδ2 cells controlled and prevented lethal Epstein–Barr virus (EBV)-transformed leukemia xenografts in immunocompromised mice (4), and *in vitro* and *in vivo* data suggested that Vδ1 cells are also specific for EBV (38, 39). In contrast to Vδ1 and Vδ2 cells, very little is known about human γδ T cells expressing other TCRγδ alleles except for indirect evidence of Vδ3 cell's immunity against CMV and HIV (40, 41). Given the multivalent nature of γδ T cells, harnessing γδ T cells populations with polyclonal TCR repertoire is attractive for adoptive immunotherapy.

## γδ T-CELL CLINICAL EXPERIENCE

Immunotherapy with γδ T cells requires their activation and expansion as they comprise only a small percentage of circulating T cells. Interleukin-2 (IL-2) and activating CD3 antibody (OKT3), commonly used for the propagation αβ T cells directly from peripheral blood mononuclear cells (PBMC), do not reliably expand γδ T cells without further manipulation and so alternative approaches are needed. Aminobisphosphonates, e.g., Zoledronic Acid (Zol), used in the treatment of bone-related diseases, e.g., osteoporosis, resulted in *in vivo* propagation of γδ T cells, and the use of aminobisphosphonates has been subsequently translated into laboratory practice to grow γδ T cells *ex vivo* (**Figure 1A**) (42, 43). Aminobisphosphonates inhibit cholesterol synthesis and result in the accumulation of phosphoantigen intermediates in the mevalonate–CoA pathway, including IPP, a ligand for Vγ9Vδ2 (44). However, only the Vγ9Vδ2 T-cell subset is reactive to cells treated with phosphoantigens (45, 46). Synthetic phosphoantigens, e.g., bromohydrin pyrophosphate (BrHPP) (47) and 2-methyl-3-butenyl-1-pyrophosphate (2M3B1PP) (48), can mimic aminobisphosphonates and stimulate Vγ9Vδ2 T cells for proliferation.

These reagents have been transitioned to the clinic for investigational treatments of cancer and HIV (**Table 1**) (49, 50). Six trials have evaluated the ability of aminobisphosphonates or BrHPP to generate *in vivo* expansions of Vγ9Vδ2 T cells to fight leukemia/lymphoma (51, 52), melanoma (52), renal cell carcinoma (RCC) (52, 53), hormone-refractory prostate cancer (HRPC) (54), breast cancer (55), and HIV (56). These trials established safety of large Vγ9Vδ2 T cell expansions *in vivo* and generated a total of nine objective responses (11.3%; $N = 80$) but no complete responses (CR) as anti-tumor therapies. Six clinical trials have used either Zol, BrHPP, or 2M3B1PP to expand autologous Vγ9Vδ2 T cells *ex vivo* and these cells were directly infused (three trials with added IL-2 infusion and three without) for treatment of RCC (57–59), non-small cell lung cancer (NSCLC) (60, 61), and colorectal cancer (CRC) (62). Direct infusion of Vγ9Vδ2 T cells was established as a safe regimen and a total of eight objective responses (11.3%; $N = 71$) were detected, including one CR (1.4%; $N = 71$) (62). Three trials have evaluated the combination of adoptive transfer of *ex vivo* expanded Vγ9Vδ2 T cells followed by Zol administration to boost their *in vivo* proliferation. Multiple myeloma (63), RCC (64), and multiple metastatic tumors (melanoma, CRC, gastrointestinal tumors, ovarian cancer, breast cancer, cervical cancer, and bone cancer) (65) were treated with this combination, which was established to be safe, and four objective responses (13.8%; $N = 29$) were observed, two of which were CRs (6.9%; $N = 29$) treating intermediate-stage RCC (64) and breast cancer (65). Thus, adoptive transfer and *in vivo* expansions of Vγ9Vδ2 T cells are safe therapeutic modalities and can result in objective clinical responses in the treatment of cancer.
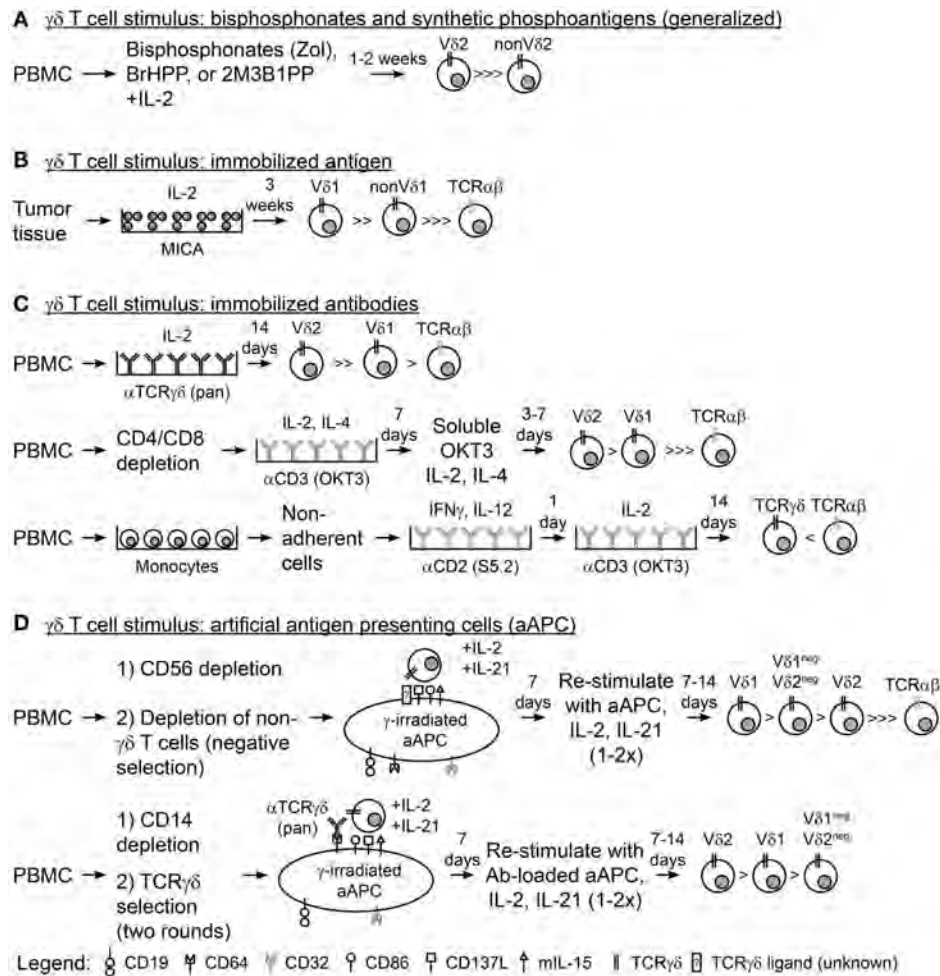
**FIGURE 1 | Methodologies for expanding γδ T cells *ex vivo*.**
**(A)** A generalized schematic for the use of aminobisphosphonates (Zol, zoledronic acid) or synthetic phosphoantigens (BrHPP, bromohydrin pyrophosphate; 2M3B1PP, 2-methyl-3-butenyl-1-pyrophosphate) and interleukin-2 (IL-2) to expand γδ T cells from peripheral blood mononuclear cells (PBMC). **(B)** Plate-bound MHC class-I chain-related (MICA) and IL-2 were used to expand γδ T cells from colon and ovarian tumor tissues. **(C)** Immobilized antibodies (Ab) were used to expand γδ T cells from PBMC in three scenarios: (top) PBMC directly stimulated with anti-pan-TCRγδ Ab and IL-2, (middle) PBMC depleted of CD4 and CD8 T cells followed by two rounds of stimulus with anti-CD3 Ab (OKT3), IL-2,

and IL-4, and (bottom) PBMC were depleted of non-adherent cells, stimulated with anti-CD2 Ab (S5.2), interferon-γ (IFNγ), and IL-12, then stimulated with OKT3 and IL-2. **(D)** Schematic for the use of artificial antigen presenting cells (aAPC) to expand γδ T cells from PBMC in two scenarios: (top) PBMC was depleted of CD56+ NK cells then of other non-γδ T cells (TCRγ/δ+ magnetic bead kit) so that γδ T cell were isolated by "negative selection" and co-cultured recursively with aAPC, IL-2, and IL-21 for 2–3 rounds of stimulation; (bottom) PBMC was depleted of CD14+ monocytes and "positively selected" with TCRγδ magnetic beads then co-cultured recursively with anti-TCRγδ Ab-loaded aAPC, IL-2, and IL-21 for 2–3 rounds of stimulation.

Allogeneic γδ T cells have also been infused but were part of heterogeneous cell populations (**Table 1**). Patients with acute myelogenous leukemia (AML) and acute lymphoblastic leukemia (ALL) were treated with αβ T cell-depleted hematopoietic stem cell transplant (HSCT), which resulted in 100 objective responses (65%; $N = 153$) with 36 durable CRs (24%; $N = 153$) (66–69). These complete remissions could be directly correlated to the elevated persistence of donor-derived Vδ1 cells in the peripheral blood of the patients, suggesting that these cells were involved in long-term clearance of leukemia. Increases in peripheral Vδ1 cells have also been correlated with CMV re-activation in patients with leukemia following allogeneic HSCT (40, 70). Most recently,

haploidentical PBMC were depleted of CD4+ and CD8+ cells using magnetic beads and were administered to patients with refractory hematological malignancies followed by Zol and IL-2 infusions (71). Three of the four patients treated experienced short-lived CRs (2, 5, and 8 months) and the other patient died of infection 6 weeks after treatment. Expansion of γδ T cells was observed the week after treatment suggesting that they may have directed the anti-tumor response. Currently, clinical trials of direct infusion of activated, homogenous populations of Vδ1 cells, or other non-Vγ9Vδ2 cells have yet to be undertaken but hold promise as future avenues of medical intervention.

**Table 1 | Clinical responses from γδ T cells.**

| Year | Treatment | Disease (N) | Total (N) | OR (%) | CR (%) | Reference |
|------|-----------|-------------|-----------|--------|--------|-----------|
| 1996 | Allogeneic HSCT depleted of αβ T cells with TBI | ALL<br>AML<br>CLL | 74 | 43/74 (58%) | 25/43 (58%) | (68) |
| 2003 | Pamidronate and IL-2 | MM (8)<br>FCL (4)<br>CLL (4)<br>MZL (2)<br>IC (1) | 19 | 3/19 (16%) | 0/19 (0%) | (51) |
| 2007 | Zol vs. Zol and IL-2 | HRPC (18) | 18 | 3/18 (17%) | 0/18 (0%) | (54) |
| 2007 | 2M3B1PP-expanded autologous Vδ2 T cells and IL-2 | RCC (7) | 7 | 3/7 (43%) | 0/7 (0%) | (57) |
| 2007 | Allogeneic HSCT depleted of αβ T cells | ALL (77)<br>AML (76) | 153 | 100/153 (65%) | 36/153 (24%) | (66) |
| 2008 | BrHPP-expanded Vδ2 T cells and IL-2 | RCC (10) | 10 | 0/10 (0%) | 0/10 (0%) | (58) |
| 2009 | Zol and IL-2 | HIV (10) | 10 | N/D | N/D | (56) |
| 2009 | Zol-expanded Vγ9Vδ2 T cells, Zol, and IL-2 | MM (6) | 6 | 0/6 (0%) | 0/6 (0%) | (63) |
| 2010 | Zol-expanded Vγ9Vδ2 T cells | NSCLC (10) | 10 | 0/10 (0%) | 0/10 (0%) | (60) |
| 2010 | Zol and IL-2 | Breast cancer (10) | 10 | 1/10 (10%) | 0/10 (0%) | (55) |
| 2010 | BrHPP-expanded Vδ2 T cells and IL-2 | RCC (18)<br>GI-cancer (4)<br>CRC (3)<br>Breast cancer (2)<br>EOC (1) | 28 | 0/28 (0%) | 0/28 (0%) | (59) |
| 2011 | Zol-expanded Vγ9Vδ2 T cells | NSCLC (15) | 15 | 0/10 (0%) | 0/10 (0%) | (61) |
| 2011 | BrHPP-expanded Vδ2 T cells, Zol, and IL-2 | RCC (11) | 11 | 1/11 (9%) | 1/11 (9%) | (64) |
| 2011 | Zol and IL-2 | RCC (12) | 12 | 0/12 (0%) | 0/12 (0%) | (53) |
| 2011 | Zol-expanded Vγ9Vδ2 T cells and Zol | Melanoma (7)<br>CRC (3)<br>GI-cancer (2)<br>EOC (2)<br>Breast cancer (2)<br>Cervical cancer (1)<br>Bone cancer (1) | 18 | 3/12 (25%) | 1/12 (8%) | (65) |
| 2012 | Zol and IL-2 | RCC (7)<br>Melanoma (6)<br>AML (8) | 21 | 2/21 (10%) | 0/21 (0%) | (52) |
| 2013 | Zol-expanded Vγ9Vδ2 T cells | CRC (6) | 6 | 5/6 (83%) | 1/6 (17%) | (62) |
| 2014 | CD4/CD8-depleted haploidentical PBMC, Zol, and IL-2 | T-NHL (1)<br>AML (1)<br>SPL (1)<br>MM (1) | 4 | 3/4 (75%) | 3/4 (75%) | (71) |

*A survey was taken of clinical trials that reported the use of aminobisphosphonates, synthetic phosphoantigens, direct infusion of ex vivo expanded γδ T cells, combinations of aminobisphosphonates/synthetic phosphoantigens/ex vivo expanded γδ T cells, and allogeneic transplants containing γδ T cells. The year reported is the year of publication. The total number (N) of each disease treated and overall patients treated with each regimen are reported. Overall responses (OR) and complete responses (CR) from these reports are listed as numbers of patients responding over total patients with frequencies of response below. The OR was pooled partial and complete responses by RECIST (when applicable and reported) or by disease-free progression (when RECIST was not applicable or reported). References to the clinical trials are included in the far right column.*

## *EX VIVO* PROPAGATION OF NON-Vγ9Vδ2 γδ T CELLS

Populations of γδ T cells outside of the Vγ9Vδ2 subset have been grown with immobilized TCRγδ agonists. Plate-bound recombinant MICA and IL-2 were used to sustain the proliferation of γδ T-cell cultures *ex vivo* from epithelial ovarian cancer and CRC tumor infiltrating lymphocytes (TILs) and resulted in high frequencies of Vδ1 cells (**Figure 1B**) (72). In addition, plate-bound pan-TCRγδ-specific antibody and IL-2 led to proliferation of both Vδ2 and Vδ1 cells (Vδ2 >> Vδ1) from peripheral blood derived from both healthy donors and patients with lung cancer or lymphoma (**Figure 1C**, top) (73, 74). Similarly, OKT3 has been used in combination with IL-2 and IL-4 to stimulate CD4/CD8-depleted T cells from healthy peripheral blood, which resulted in expansion of Vδ2 and Vδ1 cells (Vδ2 > Vδ1), albeit with reduced cell numbers compared to the TCRγδ monoclonal antibody (mAb)-stimulated cells (**Figure 1C**, middle) (75). A more complex cocktail of cytokines [IL-2, IL-12, and Interferon-γ (IFNγ)] has also been used with OKT3 and CD2-specific antibodies to expand γδ T cells, but the Vδ repertoires were not reported (**Figure 1C**, bottom) (76). Transition of these immobilized antigens and antibodies into clinical manufacture will streamline the application of these expansion strategies for γδ T cells and could be the source of clinical trials with non-Vγ9Vδ2 cells.

Highly polyclonal γδ T cells have been generated through co-culture of patient or healthy donor γδ T cells with irradiated artificial antigen presenting cells (aAPC), IL-2, and IL-21 (77–80). The aAPC (clone#4) are derived from the chronic myelogenous leukemia (CML) cell line K562 following genetic modification with T-cell co-stimulatory molecules (CD86 and CD137L), Fc receptors for antibody loading (introduced CD64 and endogenous CD32), antigens (CD19), and cytokines (a membrane-bound IL-15), and have been produced as a master cell bank (MCB) (81). This MCB is currently used in the production of αβ T cells for cancer treatments in clinical trials at MD Anderson (NCT01653717, NCT01619761, NCT00968760, and NCT01497184) (79, 82, 83). γ-irradiation of aAPC prior to co-culture with T cells subjects the aAPC to death (typically at or within 3 days) thereby reducing the risk for unintended transfer of this tumor cell line into recipients (83). Deniger et al. demonstrated that circulating γδ T cells, containing a polyclonal TCRγδ repertoire, could be isolated from healthy donor venipuncture or umbilical cord blood by "unlabeled/negative" magnetic bead selection and recursively stimulated with irradiated aAPC, IL-2, and IL-21 (**Figure 1D**, top). The aAPC-expanded γδ T cells proliferated to numbers sufficient for clinical use while maintaining the expression of most *TRDV* and *TRGV* alleles and demonstrating TCRδ surface expression of Vδ1 > Vδ1$^{neg}$Vδ2$^{neg}$ > Vδ2 (77). These polyclonal γδ T-cell cultures displayed broad tumor reactivity as they were able to lyse leukemia, ovarian cancer, pancreatic cancer, and colon cancer cells. Separation of the polyclonal cultures by TCRδ surface expression showed that each T-cell subset had anti-tumor reactivity and that a polyclonal γδ T-cell population led to the superior survival of mice with established ovarian cancer xenografts. Propagation of Vδ1$^{neg}$Vδ2$^{neg}$ cells had not been previously achieved and this was the first evidence of the functional activity of this γδ T-cell sub-population. In a similar study, Fisher et al. isolated polyclonal γδ T cells from PBMC of healthy donors or

patients with neuroblastoma by first depleting monocytes followed by "positive/labeled" selection with anti-TCRγδ-hapten antibody and anti-hapten microbeads (**Figure 1D**, bottom) (79). This study made use of the Fc receptors on the aAPC surface to load anti-TCRγδ antibody where isolated γδ T cells were co-cultured with the antibody-loaded aAPC. These expanded γδ T cells expressed multiple *TRDV* and *TRGV* alleles with surface TCRδ expression of Vδ2 > Vδ1 > Vδ1$^{neg}$Vδ2$^{neg}$. Using this mode of expansion, Vδ1 and Vδ2 were mediators of antibody-independent (AIC) and antibody-dependent cellular cytotoxicity (ADCC), respectively, to neuroblastoma tumor cells (as predicted by whether or not they expressed Fc receptor CD16). aAPC-expanded polyclonal γδ T cells could be used for anti-tumor therapies because aAPC are currently available as a clinical reagent. However, human application of aAPC/mAb-expanded γδ T cells could depend on interest in the use of the current MCB of aAPC, generation of new MCB of aAPC at institutions where there are currently none, and production of γδ T cell agonistic antibodies in good manufacturing practice (GMP) conditions. Clinical testing of these cells could potentially lead to more widespread acceptance and use of γδ T cells as adoptive cellular therapies.

Given that the aAPC can sustain the proliferation of non-Vγ9Vδ2 cells to large quantities, there is opportunity for clinical translation, laboratory testing of subsets to elucidate their functions, and correlative studies. A limiting factor in studying γδ T cells has been the lack of TCRδ and TCRγ isotype-specific antibodies outside of specificity for TCRδ1, TCRδ2, TCRγ9, and TCRδ3 (where commercially available). Mice can now be immunized to generate mAb specific for desired TCRγδ isotypes where commercial and academic use of these detection antibodies can have tangible outcomes, including diagnostic and/or prognostic profiling of γδ T cells resident within tumors. γδ T-cell clones could be generated through co-culture of single γδ T cells with aAPC, and this can facilitate studies to determine Vδ/Vγ pairing, corresponding TCRγδ ligands, and pathogenic reactivity. The ligands on the K562-derived aAPC that TCRγδ binds are not currently known. Likely candidates include IPP and MICA/B for TCRδ2 and TCRδ1, respectively (22, 35). Elucidation of these interactions could assist attempts to tailor the design of the aAPC for total γδ T-cell expansion, propagation of a particular γδ T-cell lineage, or polarization toward a certain γδ T-cell phenotype (84). As an example, CD27$^{neg}$ and CD27$^{+}$ γδ T cells are associated with IL17 and IFNγ production, respectively (85–87), leading to the conclusion that expression of CD70, the CD27 ligand, on aAPC could potentially polarize these T cells toward a desired cytokine output. Thus, aAPC could be an excellent source for the study of fundamental γδ T-cell immunobiology and could yield answers not currently accessible because of limited starting cell numbers and ineffective polyclonal expansion protocols.

## GENETIC MODIFICATION OF γδ T CELLS FOR THERAPEUTIC USE

γδ T cells are also amenable to genetic modification allowing for the introduction of genes to improve their therapeutic function. For instance, re-directed specificity of T cells can also be accomplished through the introduction of recombinant TCRs with defined antigen specificity. The conventional thought is

that transfer of TCRα/TCRβ genes into γδ T cells or transfer of TCRγ/TCRδ genes into αβ T cells would not cause mis-pairing with the TCRα/TCRβ and TCRγ/TCRδ heterodimers, thereby mitigating the risk of generating inappropriate pairings such as TCRα/TCRδ, TCRα/TCRγ, TCRβ/TCRγ, or TCRβ/TCRδ heterodimers with unknown specificity (88). This mis-pairing hypothesis was modeled in mice with the ovalbumin-specific αβ TCR OT-I, which resulted in re-directed specificity of murine γδ T cells toward ovalbumin peptide, but whether or not the TCRs were actually mis-paired was not reported (89). Vγ2Vδ2 cells have been expanded with 2M3B1PP and infected with γ-retrovirus to transduce TCRαβ chains with specificity toward MAGE-A4 peptide, but co-transduction with CD8 was required in order to transfer significant MHC Class-I-restricted recognition of MAGE-A4 peptide-pulsed tumor cells (90, 91). Similar studies have transferred αβ TCRs specific for CMV pp65 peptide or minor histocompatibility antigens into γδ T cells rendering them reactive to antigen-appropriate tumor cells (92). In contrast to the above reports of introducing αβ TCRs into γδ T cells, the Vγ9Vδ2 TCR has been transferred into αβ T cells and rendered both CD4+ and CD8+ T cells reactive to multiple tumor cell lines (93). Chemotherapy (temozolomide)-resistant γδ T cells have been generated by lentiviral transduction of (6)-alkylguanine DNA alkyltransferase into Vγ9Vδ2 cells expanded on Zol (94). Chimeric antigen receptors (CARs) can be introduced into T cells and re-direct the T cell toward a specific antigen. CARs are formed by fusing a single chain antibody to one or more T-cell intracellular signaling domains, e.g., CD3ζ, CD28, and/or CD137 (95). The antibody confers specificity through its variable regions toward a particular antigen, e.g., CD19, GD$_2$, HER2, etc., and CAR binding to the antigen transmits intracellular T-cell signals for antigen-dependent proliferation, cytokine production, and cytolysis (96, 97). Following expansion on Zol, Vγ9Vδ2 cells were efficiently transduced to express CD19- and GD$_2$-specific CARs with γ-retroviral vectors and displayed re-directed specificity toward CD19+ and GD$_2$+ tumor targets, respectively (98). Zol and γ-retroviruses engineered to transduce CD19- and GD$_2$-specific CARs are available for human application, but have not been combined in a clinical trial to date. Thus, subsets of γδ T cells are amenable to viral gene transfer to improve their therapeutic impact.

In contrast to γ-retroviruses and lentiviruses, which require cell division for efficient transduction, non-viral *Sleeping Beauty* (SB) transposition transfers genes into quiescent T cells and allows manipulation of cells that are difficult to culture *ex vivo* (99–102). SB transposase enzyme was originally derived from fish that were undergoing active transposition in their evolutionary maturation and was adapted for human application (103). In short, a DNA transposon with flanking inverted repeats and direct repeats is ligated into the human genome at TA dinucleotide repeats by the SB transposase enzyme (104). TA dinucleotide repeats are widely distributed in the human genome, yielding potential for random integration into the genome, and have been shown to be safe in regards to transgene insertion in pre-clinical studies (99, 101, 105). This is of particular importance in gene therapy as inappropriate integration at gene start sites or promoters, within exons, or even distal to genes within enhancers or repressors may cause cellular

transformation. Lentiviruses and γ-retroviruses have higher efficiency in transgene delivery than SB, but these vectors are known to integrate near genes or within genes (97). Application of SB to human clinical-grade T cells has been reduced to practice as a two DNA plasmid system, where one plasmid contains the SB transposon with the transgene of interest, e.g., CAR, and the other plasmid encodes a hyperactive SB transposase (106). Electro-transfer of the DNA plasmids by nucleofection into circulating (quiescent) PBMC results in transient expression of SB transposase that then ligates the transposon into the genome using a "cut-and-paste" mechanism. As soon as the SB transposase mRNA is degraded translation of SB transposase protein is halted, thereby negating additional transposition events. T cells with stable CAR expression can be encouraged through the co-culture of T cells on irradiated aAPC that express antigen for the CAR (83). This process, originally developed for αβ T cells, has been adapted for expression of CAR in γδ T cells (78). Resting PBMCs were electroporated with CD19-specific CAR transposon and SB11 transposase plasmids and sorted the following day to deplete non-γδ T cells with magnetic beads from the transfected mixture. Isolated γδ T cells were recursively stimulated with CD19+ aAPC along with IL-2 and IL-21, which resulted in the outgrowth of CAR+ γδ T cells with a highly polyclonal TCRγδ repertoire. Endogenous leukemia reactivity by the aAPC-expanded γδ T cells was improved through expression of CD19-specific CAR rendering these T cells bi-specific through CAR and TCRγδ. SB transposon and transposase are available as clinical reagents; therefore, clinical trials can test the safety and efficacy of bi-specific CAR+ γδ T cells.

## CONCLUDING REMARKS

Given that γδ T cells are unlikely to cause graft-versus-host disease (GVHD) because their TCR ligands (IPP, MICA, etc.) are not MHC-restricted, γδ T cells (with or without genetic modification) could be generated from healthy donors in a third party manufacturing facility and given in the allogeneic setting as an "off-the-shelf" therapeutic. Additionally, a "universal" bank of polyclonal γδ T cells could be established that was known to have high anti-tumor immunity or contain a particular set frequency of Vδ1, Vδ2, and Vδ1$^{neg}$Vδ2$^{neg}$ populations to achieve superior efficacy (66). This could have specialized application in cases where T cells were difficult to manufacture, e.g., high tumor burden in blood or after extensive systemic (lymphodepleting) chemotherapy. Polyclonal γδ T cells could also be used as front-line therapy before addition of HSCT, CAR+ T cells, TILs, etc. in order to prime the tumor microenvironment for other adaptive immune cells with broader tumor specificity or to reveal neo-tumor antigens, including somatic non-synonymous mutations expressed only in the tumor (107–109). If immunity is restored in the recipients then the 3rd party γδ T-cell graft may be rejected, but there may still be a therapeutic window before this occurs. Both pro-tumor and anti-tumor effects of γδ T cells infiltrating the tumor microenvironment have been described (110, 111), and whether or not these cells could be useful for therapy could be delineated following expansion of γδ T cells from solid tumors on aAPC, which have been shown to expand TIL (αβ T cells) from metastatic melanoma (112). Tumor lysis by γδ T cells could lead to other resident cell types, e.g., NK cells, macrophages, αβ T cells, etc., to have renewed

reactivity to the malignancy (113). Indeed, B-ALL cell lines coated with mAb were lysed by $CD16^+$ Vγ9Vδ2 cells via ADCC, and subsequently the Vγ9Vδ2 had antigen presenting cell function to generate antigen-specific $CD8^+$ αβ T cell responses to known B-ALL peptides, e.g., PAX5 (114, 115). Unknown is whether γδ T cells will be subjected to inhibition by regulatory T cells or other immunosuppressive forces. Some γδ T cells have been reported to have immunosuppressive function, and it would be of interest to identify these cells and eliminate them from the adoptive T-cell product prior to infusion (116). In summary, administration of graded doses of autologous and allogeneic, even 3rd party, γδ T cells in humans have tested and will continue to evaluate the ability of these lymphocytes to home and recycle effector function in the tumor microenvironment. Given the development of aminobisphosphonates, synthetic phosphoantigens, immobilized antigens, antibodies, and designer clinical-grade aAPC, it now appears practical to sculpt and expand γδ T cells to achieve a therapeutic effect.

## AUTHOR CONTRIBUTIONS

Drew C. Deniger, Judy S. Moyes, and Laurence J. N. Cooper wrote the manuscript.

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8-2013

# T-CELL TREATMENTS FOR SOLID AND HEMATOLOGICAL TUMORS

Drew C. Deniger

# T-CELL TREATMENTS FOR SOLID AND HEMATOLOGICAL TUMORS

by

Drew Caldwell Deniger, M.S.
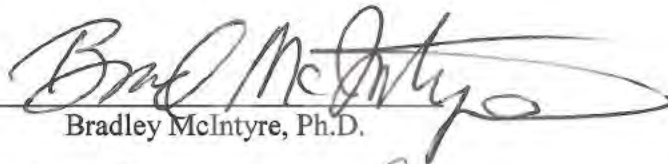
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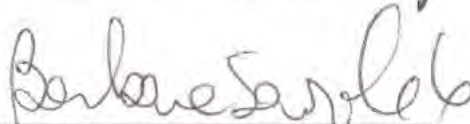
Laurence J.N. Cooper, M.D., Ph.D.; Supervisory Professor
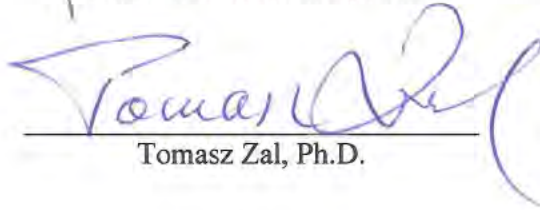
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T-CELL TREATMENTS FOR SOLID AND HEMATOLOGICAL TUMORS


A

DISSERTATION

Presented to the Faculty of
The University of Texas
Health Science Center at Houston
and
The University of Texas
MD Anderson Cancer Center
Graduate School of Biomedical Sciences
in Partial Fulfillment

of the Requirements

for the Degree of


DOCTOR OF PHILOSOPHY



by

Drew Caldwell Deniger, M.S.

Houston, Texas

August 2013

## DEDICATION

This dissertation is dedicated to one of my namesakes. He was a man I never met but have been told I would have liked, who is one of only two people in my family to have received a doctorate degree and said "much obliged" to those whom he encountered. To Robert James Caldwell, D.D.S. (Pictures enclosed)

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Many people have contributed to this work in the areas of experimental assistance, training, guidance, mentorship, cheerleading, praying, financial contributions, or combinations thereof. This list could never be totally complete, so please excuse me for any omissions.

First and foremost, I need to express my sincere appreciation for the mentorship provided by Dr. Laurence JN Cooper. He has funded, developed, supported, and fought for each of the ideas presented here and has made them into real therapies for patients with cancer. My gratitude to him is not measureable. I knew that I wanted to work for Dr. Cooper while still in my Master of Science degree program when I first heard about CARs and T cells killing tumors in my Translational Sciences class. Using the immune system to target cancer seemed like a logical and diplomatic strategy, and I was mesmerized by Dr. Cooper's enthusiasm to the field (as most people are, I believe). One rotation later, I joined the Cooper lab and have been growing T cells ever since. During my time in his lab, he taught me how to write grants, which has had its benefit in the recent past and will serve me throughout my career as a biomedical researcher, and I will remember our strategy sessions on how to translate our ideas into the clinic fondly. I can admit that the ROR1-specific T cell project was mired in roadblocks that made it seem impossible at times, but Dr. Cooper was determined to make it into a therapy for cancer patients and encouraged me to stick it out. Now it seems that these T cells will have broad applicability as cancer treatments. In the same light, I had a hair-brained idea to try and grow $\gamma\delta$ T cells, which was not my project at the time, and Dr. Cooper

was supportive of me pursuing this line of questioning. A few pilot experiments eventually resulted in two chapters of this dissertation. Last but certainly not least, he introduced me to my future boss Dr. Steven A. Rosenberg during his precious time visiting with him MD Anderson that resulted in accepting a post-doctoral fellowship at the NIH. For these things and many more – thanks!

I have also been blessed to have worked for two other mentors. First was Dr. Robert A. Davey, who was tasked with teaching me how to use the things I had learned in college to become a professional scientist. Simple practices were key to success: (i) making stock solutions to cut down on pipetting and have internal control of experiments, (ii) why moving tubes up and down on a rack allows you to remember what you've added, (iii) ask the right questions, (iv) the details matter, and (v) write everything down in detail because you will forget and you need to know. After leaving Dr. Davey's lab, I was privileged to earn my MS degree in Cancer Biology under the mentorship of Dr. Madeleine Duvic. Now I am the master of the Western blot. She asked me one day after I started my Ph.D. work in Dr. Laurence Cooper's lab, "You know what would be really great? Make one of those T cell treatments for my patients who have T cell lymphoma." That was the start of the γδ T cell project, which is a major focus of this dissertation. The idea was that γδ T cells could kill malignant αβ T cells. Although this never really happened *per se* as I had imagined it, it led to countless opportunities and may one day be a viable option for cancer treatments in lymphoma and leukemia, as well as non-hematological tumors. Ten years after first working at UTMB, I am now poised to graduate with a Doctor of Philosophy degree from the Cooper lab.

stimulatory molecules, cytokines, antigens, etc., were cloned into and expressed on primary cells and established cell lines. Also, he was very helpful with cloning strategies and molecular biology techniques. Dr. Sonny Ang brought hypoxia to the Cooper lab, assisted early work with CARs targeting ovarian cancer, and cloned CD86 and CD137L for aAPC deconstruction experiments. Dr. Marie Forget and I talked about $\gamma\delta$ T cells in melanoma and I hope to continue our collaboration while at the NCI Surgery Branch. Dr. Brian Rabinovich made lentiviral vectors that were used to make *ffLuc*-mKate virus-like particles for transduction of CAOV3 (ovarian cancer) and Kasumi-2 (B-cell ALL) cell lines. Radhika Thokala established the Kasumi-2-*ffLuc*-mKate cell line and helped with BLI. Dr. Pallavi Renkata-Manuri taught me how to do my first electroporation and was generous with early guidance on growing T cells. Dr. Hiroki Torikai helped me extensively with testing allogeneic reactivity, growing monocyte-derived dendritic cells, and experiments with umbilical cord blood. Dr. Bipulendu Jena created an anti-CD19-specific CAR idiotypic antibody that was used for sorting experiments. He and Rineka Jackson constructed the Appendix M of the ROR1-specific T cell Phase I trial. Dr. Colleen O'Connor imparted that flicking a cell pellet was the best way to prime cells for resuspension, and she talked football with me during monotonous tissue culture periods – that is a major bonus for a Texas-born native. Margaret Dawson Johnston and Matthew Figliola processed almost all blood used in these studies and did uncountable other tasks around the lab to keep it running smoothly. Ling Zhang, Cuiping Dai, and Gary Ye were also vital for lab upkeep and providing lab-wide services. Dr. Kumar Pappanaicken helped with flow cytometry for ROR1 staining and will likely do pilot studies combining ROR1-specific T cells and $\gamma\delta$

T cells for pancreatic cancer treatments. I would also like to thank Linda Lopez, Barbara Liddle, Ruby Robinson, Tiffany Tran, Beverly Smith, and Cha Davis for their assistance. It is only appropriate to end with Helen Huls – the heart and soul of the Cooper lab. Without her we are a mess – literally at times. If you need to know how to do anything in regards to tissue culture, translating lab protocols for clinical trials, or how to manage a workforce, then she is your lady. Again, it would take too long to write down everything Helen has done for me in my professional development, but suffice to say that I would not be at this place without her. To very briefly summarize, the Cooper lab has been a great place to work.

Members of my GSBS committees and other contributors have been invaluable to this process. Drs. Vidya Gopalakrishnan, Dean A Lee, Brad McIntyre, Prahlad Ram, Barbara Savoldo, Stephanie Watowich, and Tomasz Zal served on my advisory, examination, and/or supervisory committees and were instrumental in developing and challenging all ideas presented herein and in my academic development. They have also helped edit this document along with Dr. George McNamara, Dr. Spencer Stonier, Dr. Brian Friedrich, Dr. Maiti, Dr. Cooper, and Denise Crossland. Dr. Lee was kind to meet with many graduate students on Thursday night for writing advice, which was extremely useful, and was very helpful at lab meetings on Friday mornings. Dr. Thomas J Kipps was kind to give us the 4A5 monoclonal antibody specific for ROR1 and the corresponding genetic sequences in order to make the ROR1-specific CARs. Dr. William Wierda has pushed for ROR1-specific T cells for leukemia treatment, and provided primary CLL patient samples. Dr. Robert C. Bast, Jr. was gracious with his time and expertise in ovarian cancer as well as providing 12 ovarian cancer cell lines for

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Also in play were the core facilities at MD Anderson. The Immune Monitoring Core Lab (IMCL) performed and analyzed Luminex and ELISpot assays. Flow cytometry core lab assisted with FACS and mKate flow cytometry. STR fingerprinting was done by Center for General Cell Group System (CGCS) to corroborate cell line identity. I give my thanks to them for their expertise.

There have also been a number of funding agencies that have believed and supported many parts of the research in this dissertation. The GSBS Research Assistantship was awarded to me during my first 2 years of graduate school. Travel awards were generously provided by the UT-Houston GSBS, Immunology Program at UT-Houston GSBS, Society for Immunotherapy of Cancer (SITC), and American Society of Gene & Cell Therapy (ASGCT). An Excellence in Research Award was also granted by ASGCT at their 16th annual meeting. Scholarships in cancer research were given to support this dissertation by the American Legion Auxiliary (ALA) Foundation and Andrew Sowell-Wade Huggins and Cancer Answers Foundations. The Department of Defense Ovarian Cancer Research Program provided stipend and travel support in

On a personal note, there are three people I need to mention from my childhood who have molded me into the man I am today and without whose influence I would not be writing this document today. When I was 14, I had a soccer coach, Stefan, who taught me exhaustion was not a time to quit – it was at that time that you pushed harder to get what you wanted. This is a crucial skill doing lab work and keeping a high level of concentration for hours on end with no food and endless distractions. The second person was my cardiologist, Dr. Chris Wyndham, who performed a largely experimental ablation surgery to cure me of supraventricular tachycardia. Without his help I would not have been able to do exercise or compete athletically – both of which are a huge part of my personality and my education. Moreover, the experience was the primary piquing of my interest in medicine. Third, Coach Monty was my high school football coach and he made me cry many times. He wouldn't allow me to be "just a kicker" for the team and made me play offensive line in practice with guys twice (and sometimes three) times my size. Two years later, I was an all-district left tackle at a major high school in Texas standing 5 foot, 10 inches tall and weighing 150 pounds. Natural size, strength, and ability will only take you so far. The rest is up to determination and taking risks – especially when you are up against something bigger and stronger than you. What better analogy for someone trying to cure cancer?

Last, but certainly not in the list of contributors, is my family. My parents have always been very supportive and loving. They will not understand much of this document, but that has never stopped them from asking with genuine interest. My

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Cell-based therapies have demonstrated potency and efficacy as cancer treatment modalities. T cells can be dichotomized by their T cell receptor (TCR) complexes where $\alpha\beta$ T cells (95% of T cells) and $\gamma\delta$ T cells (<5% of T cells) express $\alpha/\beta$ and $\gamma/\delta$ TCR heterodimers, respectively. $\gamma\delta$ T cells have inherent anti-tumor immunity, but their use in the clinic is hampered by a lack of clinically-relevant expansion protocols. In contrast, $\alpha\beta$ T cells do not have predictable anti-tumor immunity so they can be re-directed to specific molecules on the tumor surface through introduction of tumor-specific molecules such as chimeric antigen receptors (CARs) for reproducible tumor killing. CARs are constructed with the extracellular specificity of a monoclonal antibody to a tumor antigen, e.g. CD19 or receptor tyrosine kinase-like orphan receptor-1 (ROR1), fused to intracellular T cell signaling domains (CD3$\zeta$, CD28, CD137). A comparative study was done between $\alpha\beta$ T cells re-directed with ROR1-specific CARs signaling through CD3$\zeta$ and either CD28 (ROR1RCD28) or CD137 (ROR1RCD137) in the first specific aim of this dissertation. CAR$^+$ T cells proliferated to clinically significant numbers and ROR1$^+$ tumor cells were effectively targeted and killed by both ROR1-specific CAR$^+$ T cell populations, although

ROR1RCD137 were superior to ROR1RCD28 in clearance of leukemia xenografts *in vivo*. The second specific aim focused on generating bi-specific CD19-specific CAR$^+$ γδ T cells with polyclonal TCRγδ repertoire on CD19$^+$ artificial antigen presenting cells (aAPC). Enhanced cytolysis of CD19$^+$ leukemia was observed by CAR$^+$ γδ T cells compared to CAR$^{neg}$ γδ T cells, and leukemia xenografts were significantly reduced compared to control mice *in vivo*. The third specific aim looked at the broad anti-tumor effects of polyclonal γδ T cells expanded on aAPC without CAR$^+$ T cells, where Vδ1, Vδ2, and Vδ3 populations had naïve, effector memory, and central memory phenotypes and effector function strength in the following order: Vδ2>Vδ3>Vδ1. Polyclonal γδ T cells eliminated ovarian cancer xenografts *in vivo* and increased survival compared to control mice. Thus, translating these methodologies to clinical trials will provide cancer patients novel, safe, and effective options for their treatment.

# TABLE OF CONTENTS

# LIST OF FIGURES

# LIST OF TABLES

## ABBREVIATIONS

aAPC: artificial Antigen Presenting Cell

αFR: α−Folate Receptor

αGalCer: α−galactosylceramide

APC: Antigen Presenting Cell

Ab: Antibody

ADCC: Antibody-dependent cell-mediated cytotoxicity

Ag: Antigen

ALL: Acute Lymphoblastic Leukemia

AML: Acute Myeloid Leukemia

ATCC: American Type Culture Collection

BCR: B-cell Receptor

BLI: Bioluminescence Imaging

CAIX: Carbonic anhydrase 9

CAR: Chimeric Antigen Receptor

CCL: CC Chemokine Ligands

CCR: CC Chemokine Receptors

CD: Cluster of Differentiation

CD19RCD28: CD19-specific CAR with CD28 and CD3ζ endodomains

CD19RCD137: CD19-specific CAR with CD137 and CD3ζ endodomains

CDR: Complementarity Determining Regions

CEA: Carcinoembryonic Antigen

cGMP: current Good Manufacturing Practices

CLA: Cutaneous Lymphocyte Antigen

CK1ε: Casein Kinase-1ε

CLL: Chronic Lymphocytic Leukemia

CML: Chronic Myeloid Leukemia

CMV: Cytomegalovirus

CRA: Chromium Release Assay

CREB: cAMP Response Element-Binding protein

CSF2R: Colony-Stimulating Factor 2 Receptor

CTLA4: Cytotoxic T-Lymphocyte Antigen 4

DC: Dendritic Cell

DTEA: Direct TCR Expression Array

eGFP: enhanced Green Fluorescent Protein

EGP-2: Epithelial Glycoprotein 2

EBV: Epstein-Barr Virus

EGFR: Epidermal Growth Factor Receptor

FACS: Fluorescence Activated Cell Sorting

FBP: Folate Binding Protein

FBS: Fetal Bovine Serum

FDA: Food and Drug Administration

*ffLuc*: *Firefly Luciferase*

FRA: oligonucleotides marking transposons signaling through CD137

GvHD: Graft-versus-Host Disease

HLA: Human Leukocyte Antigen

HIV: Human Immunodeficiency Virus

HSC: Hematopoietic Stem Cell

ICOS: Inducible T-cell Co-Stimulator

ICS: Intracellular Cytokine Staining

IFNγ: Interferon-γ

Ig: Immunoglobulin

IL: Interleukin

IL15/IL15Rα: fusion protein of IL15 to IL15Rα

IND: Investigational New Drug

IPP: Isopentenyl pyrophosphate

IRB: Institutional Review Board

L1-CAM: L1-Cell Adhesion Molecule

LAC: Leukocyte Activation Cocktail

LCA: Lymphocyte Code-set Array

LCL: Lymphoblastoid Cell Line

mAb: monoclonal antibody

MDACC: MD Anderson Cancer Center

MFI: Mean Fluorescence Intensity

MHC: Major Histocompatibility Complex

MICA/B: MHC Class-I Chain-related A/B

MIP1α: Macrophage Inflammatory Protein-1α

MIP1β: Macrophage Inflammatory Protein-1β

mKate: red fluorescent protein

MRD: Minimal Residual Disease

NCI: National Cancer Institute

NHL: Non-Hodgkin's Leukemia

NIH: National Institutes of Health

NKT cells: Natural Killer T cells

NSG: Mice with the NOD.*scid*.$\gamma_c^{-/-}$ genotype

OvCa: Ovarian Cancer

ORF: Open Reading Frame

PaCa: Pancreatic Cancer

PBMC: Peripheral Blood Mononuclear Cells

PCR: Polymerase Chain Reaction

PD1: Programmed Death-1

PI3K: Phosphoinositide 3-Kinase

PKC: Protein Kinase C

PMA: Phorbol 12-Myristate 13-Acetate

polyA: polyadenylation tail for mRNA transcripts

pSBSO: *Sleeping Beauty* transposon plasmid

PSCA: Prostate Stem Cell Antigen

PSMA: Prostate-specific Membrane Antigen

RAC: Recombinant DNA Advisory Committee

RANTES: Regulated on Activation, Normal T cell Expressed and Secreted

ROR1: Receptor tyrosine kinase-like Orphan Receptor-1

ROR2: Receptor tyrosine kinase-like Orphan Receptor-2

ROR1RCD28: ROR1-specific CAR with CD28 and CD3ζ endodomains

ROR1RCD137: ROR1-specific CAR with CD137 and CD3ζ endodomains

RPMI: Roswell Park Memorial Institute medium

SB: *Sleeping Beauty*

scFv: single-chain variable fragment

SCID: Severe Combined Immunodeficiency

SIM: oligonucleotides marking transposons signaling through CD28

STAT: Signal Transducer and Activator of Transcription

TAG72: Tumor-associated Glycoprotein 72

$T_C$: Cytotoxic T cell

$T_H$: Helper T cell

$T_H1$: Type 1 CD4$^+$ Helper T cell

$T_H2$: Type 2 CD4$^+$ Helper T cell

$T_H17$: Type 17 CD4$^+$ Helper T cell

$T_{REG}$: Regulatory CD4$^+$ Helper T cell

$T_{CM}$: Central memory T cell

$T_{EFF}$: Effector T cell

$T_{EM}$: Effector memory T cell

$T_{EMRA}$: Effector memory RA T cell

$T_M$: Memory T cell

$T_N$: Naïve T cell

TAA: Tumor-associated antigen

TCR: T-cell Receptor

TGFβ: Transforming Growth Factor-β

TIL: Tumor-infiltrating Lymphocytes

TFNα: Tumor Necrosis Factor-α

UCB: Umbilical Cord Blood

UCSD: University of California at San Diego

UPenn: University of Pennsylvania

Vδ1: γδ T cell expressing TCRδ1 isoform

Vδ2: γδ T cell expressing TCRδ2 isoform

Vδ3: γδ T cell expressing TCRδ3 isoform

VEGFR2: Vascular Endothelial Growth Factor Receptor-2

WBC: White Blood Cell

ZFN: Zinc Finger Nuclease

Zol: Zoledronic acid (Zometa)

# CHAPTER I

## INTRODUCTION

### I.A. Cancer

Cancer is caused by the uncontrolled and abnormal growth of cells that leads to disease and remains the second most common cause of death in the United States of America behind heart disease.(1) It is more prevalent in women than men where the median time at diagnosis is in their 60's and 70's, respectively.(2) Overall, the median age at diagnosis is 66 years old for all cancer types and more than 1.5 million people are estimated to have been diagnosed with cancer in 2012, according to the most current statistics from the National Cancer Institute (NCI) Surveillance Epidemiology and End Results (SEER; http://seer.cancer.gov/statistics). Of these diagnoses, >200,000 are represented from *each* of the three most common cancers: prostate, breast, and lung. The other groups of cancers, therefore, affect roughly 900,000 people per year in the United States, and some of the diagnoses carry dismal chances for survival. For example, roughly 22,000 women are expected to have a new diagnosis of ovarian cancer in 2013 where only 44% of them are expected to survive 5 years, and over 186,000 women are currently estimated to have a history of ovarian cancer in the United States. Similarly, greater than 48,000 new leukemia diagnoses, with 5-year overall survival rate of 56% are predicted for 2013, and more than 287,000 people in the United States have leukemia at present. Cancers can either arise from either (i) the hematopoietic compartment, i.e. bone marrow, blood, and lymphatic system, giving rise

to hematological tumors or (ii) tissues outside of the hematological systems that are generically termed solid tumors. Despite the many treatments that exist for cancer, novel therapies are desperately needed to decrease the mortality and morbidity of this disease.

I.A.1. Hematological Tumors

Hematological cancers are delineated by their hematopoietic differentiation status and the tissue from which the tumor arises. In regards to leukemia, the different types are separated first by either myeloid or lymphoid lineages and then into acute or chronic stages. Thus, they are classified as (i) acute myeloid leukemia (AML), (ii) chronic myeloid leukemia (CML), (iii) acute lymphoblastic leukemia (ALL), or (iv) chronic lymphocytic leukemia.(3) Immunotherapy targeting tumor associated antigens (TAA), e.g. CD19 or Receptor tyrosine kinase-like orphan receptor-1 (ROR1), have potential to lead to tumor regressions and, in some cases when targeting CD19, complete responses have been observed in the clinic.(4-7) The main focus of this dissertation is on developing immunotherapies for the lymphoid subsets of leukemia.

*I.A.1.a. B-cell Acute Lymphoblastic Leukemia*

The most common pediatric malignancy known is ALL but also affects many adults.(8-10) The median age at ALL diagnosis in 2012 was estimated to be 13 years old.(2) For B-cell ALL (B-ALL), tumors typically arise from the pro-B cell stage and retain

primitive characteristics without undergoing further differentiation.(11) A common subtype of B-ALL halted in normal B cell development is t(1;19) ALL, where the translocation results in an E2A-PBX1 fusion protein that functions in promoting developmental arrest and oncogenic transformation simultaneously.(12) Therapies are being actively sought after for treatment of this B-ALL group by targeting unique or dysregulated proteins resulting from aberrant E2A-PBX1 gene regulation.(13) Cytogenetics and flow cytometric staining of the tumor cell surface molecules are two key tools in the diagnosis of B-ALL, which has clinical presentation consistent of common ailments, i.e. fever, bleeding, pain, fatigue, and lethargy, but is commonly first detected due to high white blood cell counts (WBC).(14, 15) Aggressive treatment, including chemotherapy, radiation therapy, and hematopoietic stem cell transplantation (HSCT), has dramatically improved overall survival, but long-term health problems frequently arise following therapy particularly amongst children.(16, 17) More specifically, children in remission commonly develop secondary malignancies later in life, and most commonly develop AML.(18) Unfortunately, few effective treatments exist for AML. Incomplete eradication of the primary tumor can result in minimal residual disease (MRD) of the primary tumor and is also a common cause of malignancies later in life that are usually resistant to conventional therapies.(16, 19) Thus, it is of paramount importance that safe and effective therapies are developed for B-ALL patients in order to fully remove their primary tumor, reduce risk the for development of secondary tumors, and improve their expected quality of life as adults.

*I.A.1.b. T-cell Acute Lymphoblastic Leukemia*

T-cell ALL (T-ALL) accounts for less than 25% of ALL cases and has a dismal prognosis relative to B-ALL.(20)  The differentiation stage of T-ALL has importance as more immature T cells are correlated to more aggressive disease.(14, 21) Diagnosis and treatment are, in general, similar to those for B-ALL, although one unique and common clinical manifestation of T-ALL is a large mediastinal mass causing shortness of breath.(20, 22) Prognostic indicators for T-ALL response to therapy are widely sought after but are not yet predictive of response. However, particular emphasis on NOTCH mutations and chromosomal translocations has generated much enthusiasm for being able to stratify patients into potential responders and non-responders.(23, 24) As with B-ALL, MRD is a primary concern as it contributes to relapse in many cases and can be diagnosed by amplification of specific TCR alleles.(25) Currently, no adoptive T cell therapies directly targeting their neoplastic T cell counterparts exist for T-ALL. Therefore, development of T cells capable of fratricide may improve the outcomes for T-ALL patients in dire need of therapeutic intervention.

*I.A.1.c. Chronic Lymphocytic Leukemia*

In contrast to ALL, CLL occurs much later in life and is not as aggressive as ALL.(26) CLL often arises from activated or memory B cells and progresses slowly but is deadly nonetheless with a 5-year median survival.(27) Furthermore, a CLL profile with (i) alterations in chromosomes 11 or 17, (ii) unmutated immunoglobulin heavy chain ($IgV_H$) genes, (iii) expression of zeta-chain associated protein kinase-70 (ZAP70), (iv)

expression of CD38, (v) rapid doubling time of tumor lymphocytes, or (vi) increased serum $\beta_2$-microglobulin, soluble CD23, and thymidine kinase activity have been correlated with a more aggressive disease status and markedly decreased median survival.(28) CLL is generally asymptomatic and high WBC commonly results in early diagnosis that is later corroborated with cytogenetics and flow cytometry. Most current therapies are not curative and often require palliative care, but some strategies, e.g. chemotherapy, antibody therapy, and stem cell transplant, can extend survival up to multiple years.(29) T cell immunotherapy is an actively pursued therapy for CLL due to the many targetable TAA, e.g. CD19, CD20, CD23, CD52, and CD40, and monoclonal antibody therapies directed at these TAA have resulted in objective clinical responses in CLL treatments.(30) Furthermore, mAbs can be also adapted to T cell therapies in the form of chimeric antigen receptors (CARs) by linking a single chain antibody specific for the TAA to T cell intracellular activation domains.(31) Indeed, several clinical trials with CAR-based T cell therapies targeting CD19 have generated complete responses in both B-ALL and B-CLL (discussed further in **Chapter I.D.3.**).(4-7, 32) Because CLL can be sensitive to immunotherapy, it is a prime disease target for T cell treatments.

I.A.2. Solid Tumors

There are many different types of solid tumors but this dissertation will focus on generating T cell therapies for two model cancers with hopes of future applications to other solid tumors. Ovarian and pancreatic cancers were chosen because of (i) their poor

prognostic outcome, (ii) lack of efficacious T cell immunotherapies, and (iii) favorable responses targeting these tumors in initial pre-clinical tests.

*I.A.2.a. Ovarian Cancer*

Ovarian cancer (OvCa) is commonly referred to as "the most common gynecological malignancy."(33) The median age at diagnosis is 63 years old, and most patients are diagnosed in late stage (III or IV) which has a 5-year overall survival rate of 27%.(34-36) OvCa typically arises from the ovary, fallopian tube, or peritoneal cavity, and is unique in that traditional metastasis is not common outside of the intraperitoneal cavity.(37) Growth within the intraperitoneal cavity can grossly impact the ability of surrounding organs to function properly and, in some case, can be sites for local metastases. The most useful prognostic indicator for OvCa is CA125, also known as mucin 16 (MUC16), which is shed into the bloodstream and is predictive of progressive OvCa disease status.(38) Standard of care for women facing OvCa treatment is surgical resection and aggressive chemotherapy.(39, 40) Many immunotherapy approaches have been tried with few objective clinical responses.(41-44) Even though OvCa appears to have sensitivity to immunomodulation, a cell-based therapy that results in objective clinical responses has yet to be developed. As the survival rate is dismal for advanced OvCa, novel therapies are urgently needed to combat this disease.

*I.A.2.b. Pancreatic Cancer*

Pancreatic cancer (PaCa) is one of the worst cancer diagnoses because 1-year and 5-year overall survival rates are 20% and 5%, respectively.(45) It is commonly differentiated based on the anatomical location of the tumor where the tail, neck, and head of the pancreas are distinct locations and the pancreatic head is the most common site where tumors arise.(46) Similar to many of the cancer types discussed above, common health ailments, i.e. pain, weight loss, and appetite-related problems, are used in diagnosis, and patients are usually asymptomatic until metastases have already developed thereby limiting the ability of surgery to cure PaCa.(47) Diabetes is also a common diagnostic tool and is one of many risk factors, in addition to smoking, pancreatitis, genetic predisposition, and nutritional status.(46) Tumor resection dramatically improves outcome, but most cases involve metastases (liver and lymph nodes commonly) that are very difficult to control and treat with standard care.(48) Radiation and Gemcitabine is the standard of care for PaCa but elicits limited efficacy outside of palliative care.(49) Combinational approaches with other chemotherapies were also tested in clinical trials with some promising results but were not curative.(50) Perhaps the most promising results that have been generated are with vaccines (peptide, tumor lysate, or dendritic cells (DCs)) to boost resident immune responses to PaCa.(51, 52) Clinical data support that PaCa is sensitive to T cell responses and suggests that direct adoptive transfer of PaCa-reactive T cells could result in robust clinical responses.

**I.B. Tumor Associated Antigens**

The choice of which tumor associated antigen (TAA) to target is crucial for the success of the immunotherapy.(53, 54) The ideal TAA is not expressed on any normal tissues but highly expressed on the tumor cell surface. Most TAAs known thus far are cell surface glycoproteins that are involved in tumor growth or survival, e.g. growth factor receptors, that drive proliferation of the tumorigenic cells. Furthermore, optimal TAAs are often required for the growth of tumor cells meaning the cancer is dependent on the TAA, and removal or inhibition of the TAA or elimination of cells expressing the dependent TAA can lead to effective treatment. Dependence on the TAA is sought after in order to avoid antigen escape of tumor cells, i.e. no longer expressing the targeted TAA but continuing to proliferate, which can lead to relapse and disease progression.(55) Ideally, the TAA would exist on multiple tumor types to allow for targeting of many cancers with a single therapy. With these considerations in mind these studies focus on two TAAs, CD19 and ROR1, which have great promise as targets for cellular immunotherapy.

I.B.1. CD19

CD19 is a B-cell lineage-specific protein not expressed on other tissues and is, therefore, an ideal TAA for B-cell malignancies because B cells are not required for survival.(4, 6, 31, 56, 57) Similar to T cells, B cells have a B cell receptor (BCR) expressed on the cellular surface specific for a single cognate Ag.(58) Upon BCR/Ag binding, the B cell will proliferate and produce antibodies with specificity identical to

that of the BCR that are secreted into the circulation for opsonization and pathogen clearance.(59) The BCR complex is crucial for signal transduction, and is composed of CD19, CD21, and CD81, where CD19 is crucial for intracellular signaling.(60-62) CD19 is expressed from the early pro-B cell stage until memory stage and is lost as B cells differentiate into plasma cells. Because of its importance in B-cell function and persistence throughout B cell development, almost all (95%) of B-cell non-Hodgkin's leukemia (NHL) express CD19.(31) Successful removal of CD19^{+} tumors results in B-cell aplasia, which can be treated with serum immunoglobulin infusions to restore humoral immunity.(4, 6, 32, 56, 63) Thus, targeting CD19 has proven to be safe and effective means for eliminating B-cell neoplasms, albeit with diminished quality of life.

I.B.2 Receptor Tyrosine Kinase-like Orphan Receptor-1

In contrast to CD19, much less is known about ROR1, but what is known is that ROR1 (i) is a cell surface protein involved in Wnt5a signal transduction, (ii) plays a critical role in development, (iii) is no longer expressed post-parturition and is not found on almost all adult tissues, and (iv) has aberrant expression later in life on tumor cells making it a candidate TAA target.(64-67) ROR1 and its redundant partner in development, ROR2, were originally cloned and named neurotrophic tyrosine kinase receptor-related-1 and -2 (NTRKR1 and NTRKR2), respectively.(68) An analysis of the ROR1 protein structure reveals that it consists of signal peptide trailed by extracellular Ig-like C2 domain, Frizzled cysteine-rich domain (Fz-CRD), and Kringle domain that are followed by transmembrane (TM) alpha helix, intracellular protein kinase,

serine/threonine-rich domain, and proline-rich domains (**Figure 1a**). Sequence

alignment shows that ROR1 is 57% identical and 81% homologous to ROR2 where

there is homology in signal peptide (62%), Ig-like C2 (85%), Fz-CRD (93%), Kringle

(90%), TM (95%), protein kinase domain (90%), serine/threonine-rich (87%), and

proline-rich (54%) domains between the two proteins (**Figure 1b**). Single and double

knockout mice for ROR1 and ROR2 were established that had multiple developmental

problems leading to death shortly after birth.(69, 70) More specifically, ROR1$^{-/-}$ mice

died of respiratory distress following birth, while ROR2$^{-/-}$ mice died of more advanced

cardiovascular problems as well as skeletal abnormalities, and ROR1$^{-/-}$ROR2$^{-/-}$ double

knockout mice had exacerbated disease including transposition of the great arteries,

pubic bone dysplasia, and sternal defects. Furthermore, ROR2 continues to be critical

for skeletal development during life as autosomal recessive diseases resulting in bone

dysmorphia and have been mapped to ROR2 gene mutations (chromosome 9q22) but

not ROR1 gene (chromosome 1p32-31).(71-74) To date, ROR1 has not been linked to

inherited genetic disease in adults, indicating that its major roles are only in fetal

development. In 2008, three independent investigators published reports of ROR1

expression in tumors, and each described ROR1 expression in ~95% of CLL patients

with confirmation of absent expression on most normal tissues.(65, 75, 76)

Subsequently, ROR1 has been detected in breast cancer, pancreatic cancer, ovarian

cancer, melanoma, gastric carcinoma, non-small cell lung cancer, t(1;19) B-ALL, and

mantle cell lymphoma, but some reports indicate that cytosolic expression of ROR1

exists in some tissues and that there may be surface expression on hematogones (normal

B cell developmental precursors), the pancreas, and adipose tissue.(13, 66, 67, 77-81)

The discovery of ROR1 expression on tumor cell lines enabled a number of biochemical studies to determine the role of ROR1 in neoplastic transformation. IL6 leads to transcriptional activation of signal transducer and activator of transcription-3 (STAT3) that then increases gene expression of ROR1 transcripts, which may give insight to a potential autocrine or paracrine loop for oncogenic transformation and/or disease progression.(82) Wnt5a binding of ROR1 (presumably to the Fz-CRD) leads to casein kinase-1ε (CK1ε) activation of phosphoinositol-3 kinase (PI3K) that phosphorylates Akt and results in activation of the transcriptional activator cAMP-response-element-binding protein (CREB), which upregulates genes important for proliferation and, thus, is likely to result in oncogenic transformation (**Figure 1c**).(67, 79) The discovery of ROR1 on tumor cells is relatively new, so other signaling pathways have not been elucidated and direct targeting of ROR1 in humans has not been tested to date. Nonetheless, all indications suggest that ROR1 is an ideal TAA target for cellular immunotherapy with broad applicability, and immunotherapies targeting ROR1 in humans will be the ultimate test of its safety as a TAA.
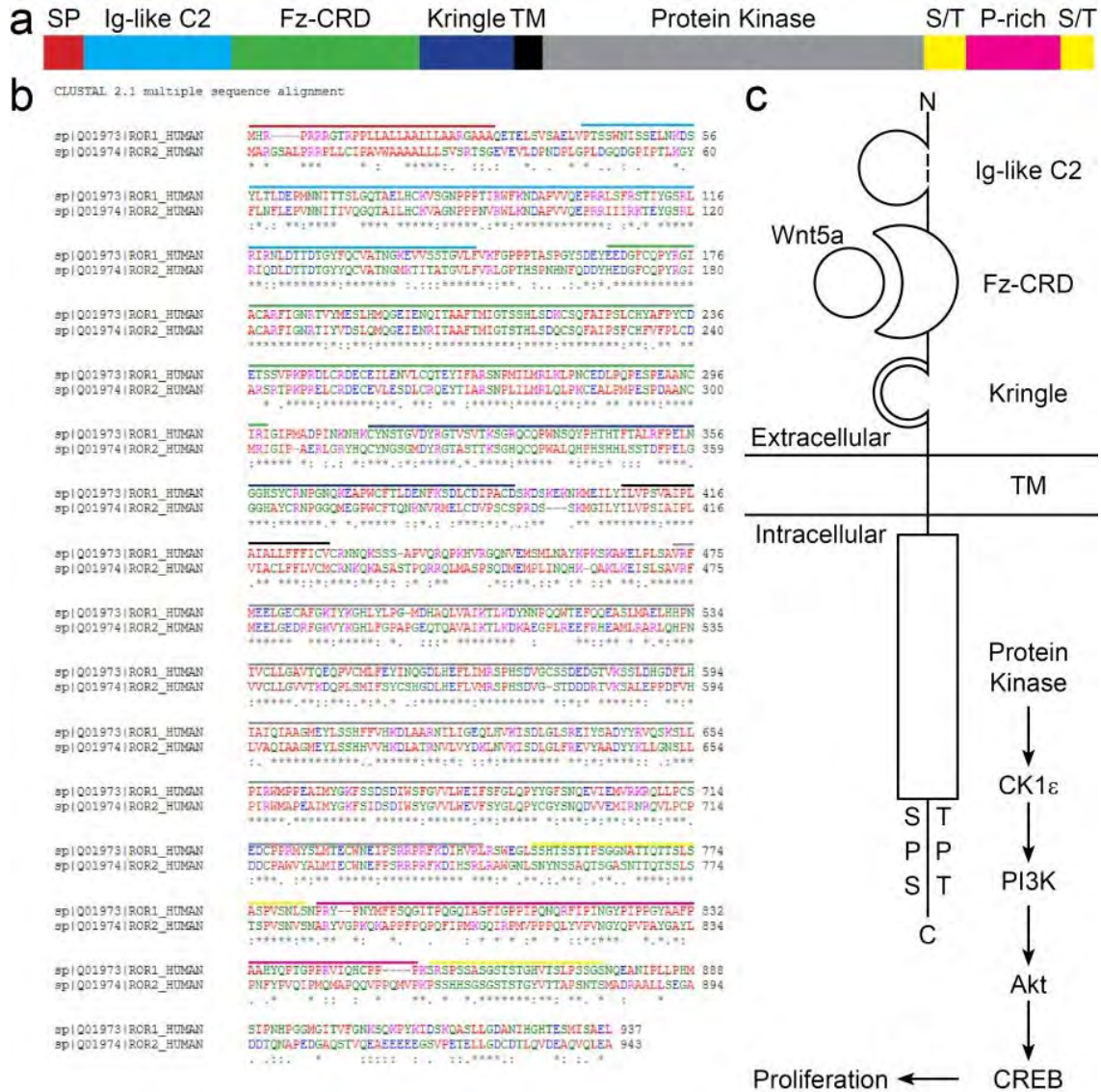
**Figure 1. ROR1 Protein Structure.** (**a**) Diagram of protein sequence of ROR1 protein domains where abbreviations are as follows: SP; signal peptide, Fz-CRD; Frizzled cysteine-rich domain, TM; transmembrane alpha helix, S/T; serine/threonine-rich domain, P-rich; proline-rich domain. (**b**) Sequence alignment between ROR1 and ROR2 proteins by ClustalOmega (http://www.ebi.ac.uk/Tools/msa/clustalo/). Lines above text correspond to colors in (**a**), (*) describes identical amino acids, (:) denotes analogous closely related amino acids, and (.) describes similar amino acids. (**c**) Diagram for ROR1 protein structure in the cellular membrane where Wnt5a binding Fz-CRD leads to the following signal transduction pathway: casein kinase-1ε (CK1ε) → phosphoinositol-3 kinase (PI3K) → Akt → cAMP-response-element-binding protein (CREB) → transcriptional activation of genes for proliferation.

**I.C. T cell Immunity**

The immune system is critical for pathogen clearance and prevention of disease. It is broadly partitioned into innate and adaptive immune systems, but interplay between innate and adaptive immunity is essential to an effective immune response.(83-86) The innate immune system is composed of many cell types, e.g. macrophages, natural killer (NK) cells, that have broad ranges of specificity to pathogens to remove them upon their primary encounter, and therefore serve as the first line of defense.(87) In contrast, the adaptive immune system is highly specific for a particular part of a pathogen and develops as a secondary and long-lasting response to a individual pathogen. The two major sections of adaptive immunity are the cellular and humoral immune systems.(88) B cells mediate humoral immunity primarily through the production of antibodies (Ab), which coat the surface of pathogens to label them as foreign for direct lysis through complement activation, which forms holes in the membrane thereby destroying the target cells, or by phagocytosis and elimination during the process known as opsonization.(89) In contrast, T cells mediate cellular immunity through direct contact with their target and either directly or indirectly mediate destruction of the pathogenic cell. T cells are typically dichotomized into helper ($T_H$) or cytotoxic/killer ($T_C$) T cells based on their expression of CD4 and CD8, respectively.(90) The combined interaction of these components of the adaptive immune system allow for its unique characteristics of (i) generating highly specific responses to pathogens, (ii) memory formation for more rapid and stronger responses to pathogens upon a repeated or secondary exposure, and (iii) adaptation to increase sensitivity through maturation.(88) Because T cells can exert

direct cellular cytotoxicity and create memory responses, they have been used successfully to target and kill cancer cells.

## I.C.1. αβ T cells

The quintessential T cell lineage is the αβ T cell subset, which comprises up to 95-99% of circulating T cells, and are the object of most canonical T cell paradigms.(58) In addition to staining for either CD4 or CD8, these T cells are typically identified by co-staining with CD3 and their αβ T-cell receptor (TCRαβ). Effector functions are endowed upon αβ T cells through an extensive educational process that results in a unique specificity to an antigen and a corresponding response in the form of T cell help (CD4) or cytolysis (CD8). Therefore, it is important to understand the nuances of αβ T cell development and education in order to maximize their impact in adoptive immunotherapy.

### I.C.1.a. T-cell Receptor Genetics

TCRs are subjected to genetic rearrangement events during development to randomly arrange distinct gene segments into an extremely high number of combinations and thus corresponding antigen affinities.(91) Four TCR loci, i.e. TCRα, TCRβ, TCRγ, and TCRδ exist in the human genome, which lead to two distinct T cell lineages based on TCR pairing.(92) More specifically, the αβ T cell lineage is defined by the pairing of TCRα and TCRβ chains whereas the γδ T cell lineage is defined by T cells expressing

TCRγ and TCRδ heterodimers. Each TCR allele is further compartmentalized into variable (V), diversity (D), junction (J), and constant (C) regions.(93) TCRα and TCRγ genes have V and J regions while TCRβ and TCRδ genes have V, D, and J regions and all TCRs contain C regions (**Figure 2**). Each specific region is termed based on its region and origin, i.e. Vα describes the variable region from the alpha locus or Jδ describes the junction region from the delta locus. The V regions contain complementarity determining regions (CDR) that confer high degrees of antigen specificity, and are therefore important for defining T cell affinity.(94) These V, D (where applicable), J, and C segments are recombined into unique combinations in each T cell during T cell development in a process known as V(D)J recombination.(95, 96) The TCRγ (Gene ID: 6965) and TCRβ (Gene ID: 6957) loci are in distinct locations at 7p14 and 7q34, respectively, but TCRδ locus (Gene ID: 6964) exists within the TCRα (Gene ID: 6955) locus at 14q11.2 (**Figure 2**). Upon V(D)J recombination of the Vα and Jα, the entire δ-chain locus is deleted from the T cell genome in a T-cell receptor excision circle (TREC).(97) Thus, once the α-chain locus has recombined for a particular T cell, it can no longer become a γδ T cell. Programmed mutation of the T cell germline DNA allows for unbiased generation of many TCR specificities for extremely high combinational probabilities (at least $10^{16}$ possible combinations for αβ T cells) for binding any potential foreign pathogen.(98) It is in this random genetic process through which T cells acquire exquisite abilities to mediate cellular immunity.
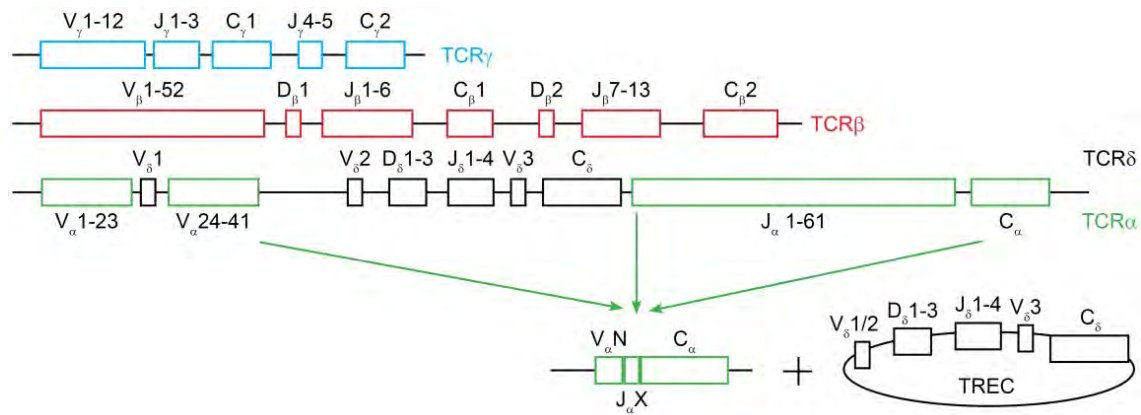
**Figure 2. Genetic Loci for TCR alleles.** Simplified schematic of exons encoding V, J, and C regions with D regions for β and δ chains for TCRγ (blue), TCRβ (red), TCRα (green), and TCRδ (black). V(D)J recombination of Vα, Jα, and Cα results in excision of the TCRδ locus in a T cell Receptor Excision Circle (TREC).

*I.C.1.b. αβ T cell Development*

The thymus is crucial for T cell development as it is the location for both V(D)J recombination and thymic selection. Thymic selection is important for maintaining central tolerance by eliminating poorly-reactive T cells and over-reactive T cells from the T cell pool by neglect and negative selection, respectively, following V(D)J recombination.(99, 100) Positive selection only allows for T cells with intermediate reactivity to their antigen to be released into the periphery.(101, 102) Thymic selection is carried out by thymic cortical epithelial cells which express high levels of major histocompatibility complex (MHC) molecules along with a wide array of proteins, including self-antigens, that are then processed and presented in the context of MHC on the epithelial cell surface.(103, 104) Both MHC Class-I (MHC-I) and Class-II (MHC-II) are expressed by the thymic cortical epithelial cells to stimulate CD8 and CD4 T cells, respectively. The developing T cells express both CD4 and CD8 in the thymus, and based on their TCRαβ binding affinity to either MHC-I or MHC-II and subsequent TCRαβ signaling they will become single positive for either CD8 or CD4, respectively.(105, 106) In this way, both affinity and peripheral T cell function is acquired in the thymic cortex.

*I.C.1.c. αβ T cell Activation*

T cells need to escape the thymus, encounter their corresponding antigen, and have a licensing event towards the antigen in order to become functionally responsive. At least two signals are required for T cell activation but 3 total signals are ideal for full T cell

activation.(107-109) Signal 1 comes from TCR$\alpha\beta$ interaction with MHC/peptide complexes mediated by CD4 or CD8 co-receptors.(110) However, the intracellular domain of TCR is very short and not able to generate its own intracellular signal. Signaling comes from CD3 molecules that are bound to TCR in the transmembrane through non-covalent interactions.(111) A complex of CD3 subunits surrounds the TCR composed of CD3$\gamma$/CD3$\epsilon$ and CD3$\delta$/CD3$\epsilon$ heterodimers and CD3$\zeta$/CD3$\zeta$ homodimer. Each of the CD3$\gamma$, CD3$\delta$, and CD3$\epsilon$ subunits has an immunoreceptor tyrosine activation domain (ITAM) motif and the CD3$\zeta$ subunit has three ITAM motifs for a total of ten ITAMs surrounding each TCR. Upon TCR$\alpha\beta$ binding to peptide/MHC complex, co-receptors (CD4 or CD8) bind to the constant regions of MHC and begin the signaling process through Lck and Fyn phosphorylation of tyrosine (p-Tyr) residues on the ITAMs.(112) Then ZAP70 can bind to p-Tyr through SH2 domains and becomes activated by Lck. Activated ZAP70 leads to a cascade of downstream activation events resulting in transcriptional and post-translational modifications for the molecules responsible for T cell proliferation and differentiation.(113) However, only receiving signal 1 will lead to functional unresponsiveness otherwise known as anergy.(114, 115) Therefore, the second signal is required and is termed co-stimulation. Examples of activating co-stimulatory molecules expressed on the T cell surface are CD27, CD28, and CD137 (41BB), which bind to CD70, CD80/CD86, and CD137L (41BB-L), respectively, expressed on the antigen presenting cell (APC).(116-118) Some co-stimulatory molecules are inhibitory, e.g. cytotoxic T-lymphocyte antigen-4 (CTLA4) and programmed death-1 (PD1), for immune regulatory purposes.(119) Dendritic cells (DCs) are professional APCs because of their ability to process and present a wide

milieu of peptides, high expression of MHC molecules, and expression of co-stimulatory molecules.(120) DCs are present in tissues and following activation by the innate immune system to foreign antigens/pathogens, they migrate to secondary lymphoid organs to present their environmental data and license T cells to fight the pathogens.(121, 122) It is also important to note that cytokine support, e.g. interleukin-12 (IL12), IL15, and type I interferon (IFN), is generally regarded as signal 3 for T cell activation.(123) In summary, the combination of (i) TCR$\alpha\beta$ engagement with MHC/peptide complex with appropriate co-receptor (CD4 or CD8) binding to MHC, (ii) co-stimulation, and (iii) cytokine support licenses T cells to find their corresponding antigen expressed on damaged or pathogenic cells and to eliminate those cells.

### I.C.1.d. CD4$^+$ $\alpha\beta$ T cell Subsets

CD4$^+$ T cell subsets are numerous and typically described by the effector cytokines they release, and they can be stratified into $T_H0$ (naïve), $T_H1$, $T_H2$, $T_H17$, regulatory T cells ($T_{REG}$), and natural killer T (NKT) cells.(124) Naïve $T_H0$ cells can be polarized to differentiate based on environmental cues that then translate into distinct transcriptional programs and result in lineage commitment.(125) $T_H1$ encourage inflammation and help promote CD8 memory responses by producing IL2, IL12, interferon-$\gamma$ (IFN$\gamma$), and tumor necrosis factor-$\alpha$ (TNF$\alpha$) while $T_H2$ cells inhibit inflammatory $T_C$ response and foster humoral immunity by secreting IL4, IL5, IL6, and IL10.(126) The primary role of $T_H17$ cells is to enhance neutrophil responses, and these cells are most often characterized by their ability to produce IL17.(127) There is plasticity between $T_H17$ cells and $T_{REG}$ cells as both require transforming growth factor-$\beta$ (TGF$\beta$) but addition

of IL6 polarizes towards $T_H17$ lineage. $T_{REG}$ cells are infrequent and can exert strong blockades against other T cell effector functions through both cell-to-cell contact mechanisms and through production of IL10 and TGFβ.(128) Thus, they are critical for maintaining peripheral tolerance, and when dysregulated can contribute to diseases such as cancer (in the case of overactive $T_{REGS}$) or autoimmune disorders (in the case of underactive $T_{REGS}$). An extremely rare subset of CD4$^+$ T cells are NKT cells, which express invariant TCRαβ alleles, e.g. Vα24/Jα18 with Vβ11, and are known to produce both $T_H1$ and $T_H2$ cytokines.(129) The best described antigen for NKT cells is α-galactosylceramide (αGalCer) presented to NKT cells in the context of CD1d, a non-classical MHC molecule, which leads to NKT expansion and effector function, but the "natural" ligands for NKT in humans are not fully known to date.(130) Some NKT cells express CD8 instead of CD4 and others express neither co-receptor, but their roles are less well known. In aggregate, CD4$^+$ T cells are an important arm of the cellular immune response and can generate a wide range of effects towards eliminating pathogens.

*I.C.1.e. CD8$^+$ αβ T cell Subsets*

In contrast to CD4$^+$ T cell subsets, CD8$^+$ T cells subsets are usually defined in terms of their memory response from previous encounters with antigens.(131) As mediators of direct cellular cytotoxicity, CD8$^+$ T cell memory responses are commonly studied in the context of pathogenic infection or in the context of long-lived tumor-reactive T cells.(132-134) After antigen exposure, naïve T cells ($T_N$) proliferate rapidly and exert

cytotoxicity as effector T cells ($T_{EFF}$). The large numbers of antigen-specific T cells then needs to be reduced as to not increase the total peripheral T cell pool each time a pathogen elicits a response, so there is a contraction phase marked by $T_{EFF}$ sensitivity to extrinsic apoptosis. However, the numbers of antigen-specific cells surviving the contraction phase are greater than the initial antigen-specific T cell pool so that exposure to the same pathogen will result in a faster and stronger attack on the pathogen. These remaining cells are termed memory T cells (**Figure 3**). Three memory T cell subsets have been described and are called central memory ($T_{CM}$), effector memory ($T_{EM}$), and effector memory RA ($T_{EMRA}$) T cells.(135) $T_N$ express CD45RA, CD27, CD28, and CCR7 where CD45RA expression is lost on both $T_{CM}$ and $T_{EM}$ but is re-expressed on $T_{EMRA}$ without CD27, CD28, and CCR7. The $T_{EM}$ and $T_{CM}$ groups can be distinguished by CD28 and CCR7 where the former expresses neither and the latter expresses both. $T_{CM}$ cells have the greatest proliferative capacity with limited effector functions and serve as long-lasting antigen-specific pools. In contrast, $T_{EM}$ have immediate effector functions, limited replicative capacity relative to $T_{CM}$, and serve as the main memory cytotoxicity mediators.(136) Lastly, $T_{EMRA}$ cells are terminally differentiated cells that have effector functions without much proliferative capacity. Even though CD4$^+$ T cells are not typically stratified in this manner, memory populations have been detected that could produce cytokines following subsequent antigen exposure.(137, 138) Furthermore, CD4$^+$ T cells are necessary for generating CD8$^+$ T cell memory, suggesting that even though they may not fit into clear subsets they are present and required for memory cytotoxicity.(139) The application of these groupings to cancer immunotherapy also comes with caveats due to the high degree of

differences in their disease pathologies, i.e. virus versus cancer. CD27 expressed on $T_N$, $T_{CM}$, and $T_{EM}$ was correlated with greatest responses in cancer immunotherapy, and can be used to predict therapeutic efficacy.(134) While immediate effector function towards cancer in adoptive T cell immunotherapies is desired, it appears that $T_N$ and $T_{CM}$ cells are better for this particular task.(131) Generation of persistent $CD8^+$ populations with memory to the tumor, therefore, is an important consideration for immunotherapy efficacy.
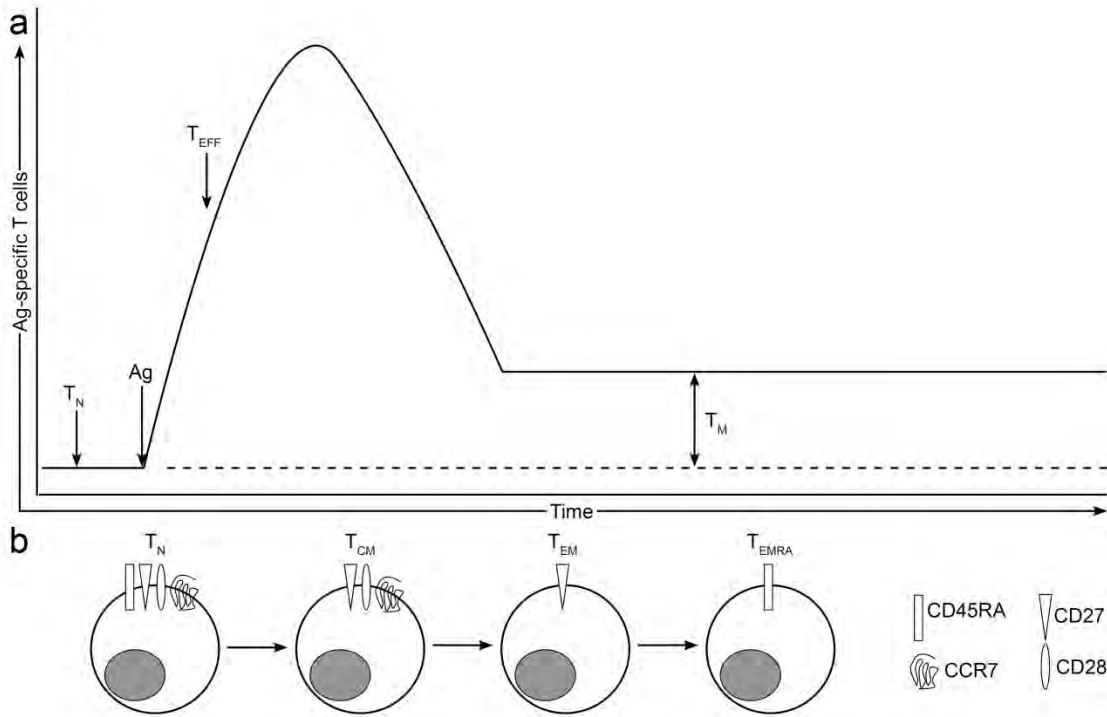
**Figure 3. CD8$^+$ Memory T cell Subsets.** (**a**) Limited quantities of antigen-specific naïve T cell (T$_N$) pool exist prior to exposure to antigen (Ag). Upon Ag contact, massive Ag-specific T cell proliferation occurs in the effector T cell (T$_{EFF}$), which is followed by apoptotic contraction phase. Memory T cells (T$_M$) are developed from the increase in Ag-specific T cell population relative to the T$_N$ starting population. (**b**) Prior to Ag exposure T$_N$ cells express CD45RA, CD27, CD28, and CCR7 where CD45RA expression is lost in the formation of T$_{CM}$ and both CD28 and CCR7 are lost with T$_{EM}$ cells. Terminally differentiated T$_{EMRA}$ cells lose CD27 expression and express CR45RA again.

I.C.2. γδ T cells

γδ T cells are a completely separate T cell lineage from αβ T cells, and γδ T cells have both innate and adaptive immune cell functions.(140) In contrast to αβ T cells, γδ T cells have predictable inherent anti-tumor immunity mediated directly through their TCR.(141) However, γδ T cells comprise only 1 – 5% of the circulating T cell repertoire, making them difficult to work with because of a relative lack of robust protocols for polyclonal γδ T cell expansion and their infrequent quantities in peripheral blood.(142, 143) They are identified by co-expression of $CD3^+TCR\gamma\delta^+$ where expression of CD4 or CD8 is rare, and can be stratified into Vδ1, Vδ2, and Vδ3 subsets based on TCRγδ alleles.(144) Targets of γδ T cells include tumor cells, viruses, bacteria, mycobacteria, and cell stress-associated proteins.(145, 146) Therefore, γδ T cells are a promising T cell immunotherapy option despite their limited frequencies in blood if they can be expanded.

*I.C.2.a. Unique Characteristics of γδ T cells*

There are three variable TCRδ chains and 14 variable TCRγ chains expressed in humans, and fewer unique TCRγδ combinations are observed in γδ T cells compared to the immense combinational diversity seen with αβ T cells following V(D)J recombination.(92, 144) Expression of TCRγδ heterodimers on the T cell surface in the thymus inhibits recombination of β-chain locus during the $CD4^{neg}CD8^{neg}$ stage thereby committing the T cell to the γδ T cell lineage.(147) This double negative status is often

maintained after exit from the thymus, most likely because TCRγδ recognizes antigens outside of MHC-restriction in many cases, making co-receptor expression dispensable for function and endowing them with an ability to recognize antigens outside of the signaling constraints imposed by classical thymic selection.(148) However, the thymus is not required for all γδ T cell development, as many of these γδ T cells take up residence in peripheral tissues and exhibit immediate effector functions against pathogens.(149) Resident γδ T cells can be found in the mucosa, tongue, vagina, intestine, lung, liver, and skin and can comprise up to 50% of the T cell populations in intestinal epithelial lymphocytes (IEL).(144, 150) In contrast, circulating γδ T cells can be found in the blood and lymphoid organs, and are canonically dominated by γδ T cells expressing Vδ2 TCR isotype (called Vδ2 cells) with few γδ T cells expressing the Vδ1 TCR isotype (called Vδ1 cells) that are more frequently associated with resident γδ T cells.(146) Moreover, Vδ2 cells most commonly pair with Vγ9, but Vδ1 and Vδ3 have broad γ-chain pairing potential.(141, 146) Therefore, the location of γδ T cells can lead to their subset diversity and effector functions that can be mediated through specific combinations of γ and δ TCR chains to recognize pathogens upon encounter in their resident or circulating locations.

*I.C.2.b. Vδ1 γδ T cells*

Vδ1 cells have a wide range of effector functions and are located in a variety of anatomical locations.(151) They can, theoretically, pair with any of the TCRγ chains, and there are a variety of known ligands for Vδ1 cells.(140) In fact, the crystal structure

of Vγ1Vδ1 has been solved in combination with one of its antigens, MHC Class-I chain-related A (MICA).(152, 153) Cellular stress and/or viral infection result in MICA and its analog, MICB, to become expressed on the stressed/infected cell's surface, so MICA/B is commonly present on tumor cell surface.(154) MICA is also recognized by NKG2D, a receptor expressed by γδ T cells, NK cells, and, less frequently, αβ T cells.(155) Other non-classical MHC molecules and cell stress proteins are also recognized by γδ T cells. For instance, Vγ4Vδ1 T cells have been shown to have specificity towards heat shock proteins and the non-classical MHC molecule CD1d.(156) Heat shock proteins are commonly over-expressed in tumor cells to handle their high protein translation loads.(157) The CD1d molecule is best described in its ability to expand NKT αβ T cells, but γδ T cells have also been described to have direct NKT-like functions, enhance NKT αβ T cells reactivity to αGalCer, and have even been shown to have specificity to cardiolipin with CD1d.(158-160) Also, murine Vγ5Vδ1 cells are well described in their ability to serve as dendritic epidermal T cells (DETCs) with APC function.(161-163) Lastly, correlative studies have implicated Vδ1 T cells to have immunity towards cytomegalovirus (CMV) and human immunodeficiency virus (HIV).(164, 165) In aggregate, Vδ1 cells have immunity towards microbial pathogens, have antigen presenting capabilities, and can target proteins expressed on the tumor surface.

*I.C.2.c. Vδ2 γδ T cells*

The most extensively studied subset of γδ T cells is the Vδ2 lineage, which similar to Vδ1 cells, recognize microbial pathogens, serve as APCs, and target cell-stress proteins expressed on tumor cells.(141, 166) Bacterial alkylamines and *Listeria monocytogenes* are recognized by Vδ2 cells when paired with Vγ2.(167-169) In contrast to Vδ1, a strong preference towards Vδ2 heterodimerizing with Vγ9 has been well documented. Vγ9Vδ2 cells have been shown to react to phospho-antigens (isopentenyl pyrophosphate; IPP), $F_1$-ATPase expressed on the cell surface, and *Mycobacterium tuberculosis*.(170-172) Furthermore, Vγ9Vδ2 cells are reactive to cells treated with aminobisphosphonates, e.g. Zoledronic Acid (Zol), which is the only current means of propagating γδ T cells *ex vivo* in the clinic.(173, 174) Aminobisphosphonates inhibit cholesterol synthesis and build up intermediates in the mevalonate-CoA pathway, including IPP, which is a ligand for Vγ9Vδ2.(175) This process was serendipitously discovered when patients with bone disorders who were treated with aminobisphosphonates to resume bone growth experienced large *in vivo* expansions of Vγ9Vδ2 T cells, and aminobisphosphonates methods were subsequently translated into laboratory practice to expand Vγ9Vδ2 cells *ex vivo*.(176) Thus, Vδ2 cells are the *only* γδ T cells that have been used for adoptive T cell therapy. Utility of the Vδ1 and Vδ3 lineages is appealing, but there are no current means to rapidly expand them to clinically-significant numbers and the existing polyclonal γδ T cell population is too few in number for direct infusion. Nonetheless, numerous clinical trials treating cancer patients with (i) infusions of Zol for *in vivo* Vγ9Vδ2 expansions and/or (ii) infusions of

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*ex vivo* expanded Vγ9Vδ2 cells have generated objective clinical responses but complete responses have been unpredictable and have not always been directly correlated to the Vγ9Vδ2 cells.(177-182) Thus, the extensive work studying Vδ2 cells has generated much interest in using γδ T cells for adoptive immunotherapy.

*I.C.2.d. Vδ3 γδ T cells*

In contrast to Vδ1 and Vδ2 cells, very little is known about γδ T cells expressing Vδ3 TCR alleles (called Vδ3 cells). The limited quantities in peripheral blood and lack of commercially available reagents for Vδ3 inhibit attempts to study this subset. Vδ3 cells are indirectly correlated with CMV and HIV immune responses, but nothing is known about their anti-tumor immunity.(165, 183) Developing a means with which to study this lineage could have important scientific and clinical significance.

**I.D. Chimeric Antigen Receptors**

Chimeric Antigen Receptors (CARs) re-direct T cells to antigens independent of their endogenous TCR specificity.(184, 185) These recombinant molecules contain in order from N-terminus to C-terminus: (i) a single chain variable fragment (scFv) derived from a monoclonal antibody with specificity to a TAA, (ii) an extracellular stalk, (iii) a transmembrane domain, and (iv) T-cell signaling endodomains (**Figure 4**). Binding of the scFv to its corresponding TAA leads to T cell activation resulting in proliferation,

cytokine release, and cytotoxicity.(186) Thus, CAR$^+$ T cells are re-directed to TAA outside of their thymically-selected affinities.

I.D.1. CAR Generations

Successive modifications to the design of CARs have improved their ability to re-direct T cells to TAAs.(187) CAR technology was invented by Dr. Zelig Eshhar (Weizmann Institute of Science, Rehovot, Israel) in 1989, and the original CAR differed from the more modern CARs by (i) having only CD3ζ and (ii) TCR constant domain scaffold.(188) Second generation CARs have shown the most efficacy in re-directing T cells and are superior to first generation CARs by adding in a co-stimulatory endodomain, e.g. CD28 or CD137 (41BB), to supplement CD3ζ signaling strength present in both generations (**Figure 4**).(189-193) Third generation CARs, therefore, contain three endodomains, and the most common combination has been CD28, CD137, and CD3ζ.(194-196) The order of endodomains does appear to have importance in the ability to stimulate the T cell in both second and third generation CARs, where CD3ζ works best at a position most distal to the membrane.(192, 197) The scaffold sequence used has the most difference between investigators where IgG4 constant regions (used in this dissertation), CD8α, no stalk, and flexible spacers have been used successfully.(13, 32, 192, 193, 198, 199) Although there exist some differences between groups in their CAR-modified T cell products in tumor killing, CARs in general have been shown as a consistent and effective means to target desired antigens and change the T cell response outside of their endogenous specificity.
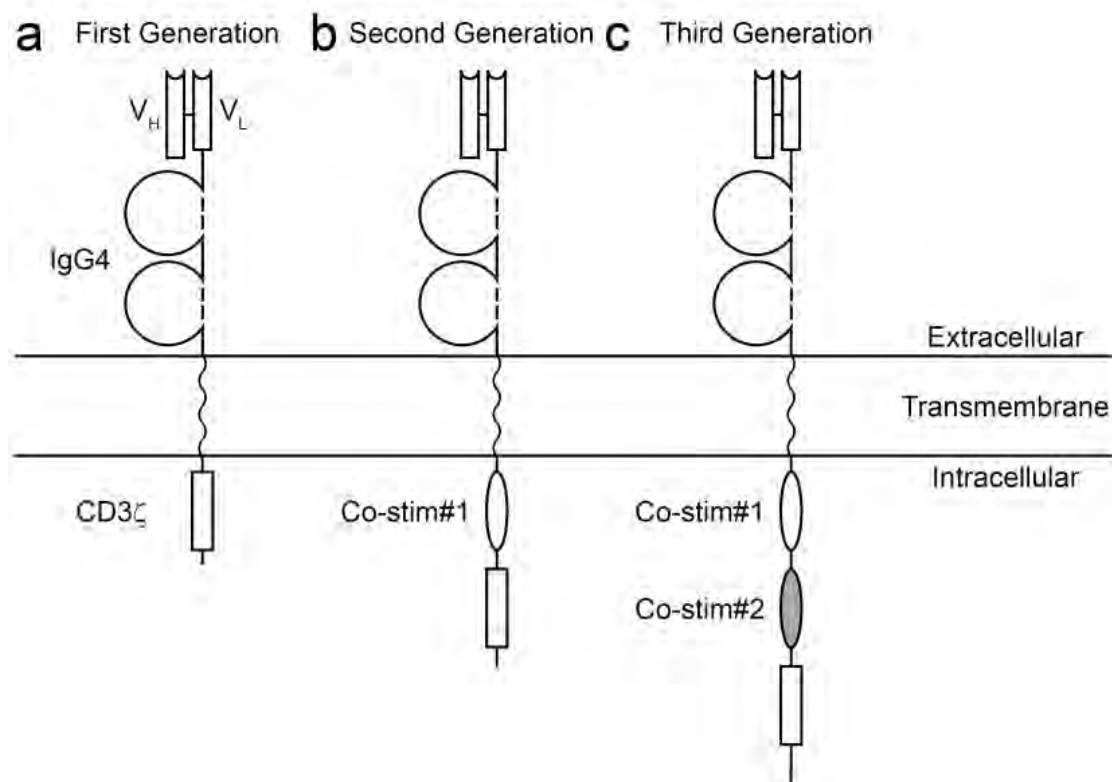
**Figure 4. Schematic Representation of CARs.** (**a**) First generation CARs were constructed with single chain variable fragments (scFv) composed of heavy ($V_H$) and light ($V_L$) variable fragments from monoclonal antibodies specific for TAA, followed by IgG4 constant region (CH2 and CH3 domains displayed), a transmembrane α-helix, and CD3ζ signaling endodomain. (**b**) Second generation CARs added a co-stimulatory domain, e.g. CD28 or CD137, between CD3ζ and transmembrane domain. (**c**) Third generation CARs use two co-stimulatory domains upstream of CD3ζ.

I.D.2. Tumor-associated Antigens Targeted with CARs

Effective targeting of different TAAs using CAR-modified T cells has generated enthusiasm around CAR-based immunotherapies. B-cell malignancies have been targeted with CARs specific for ROR1, κ-light chain, CD19, CD20, CD22, CD23, and CD30, which are all confined to the hematopoietic compartment and are not expressed on solid tissues.(57, 77, 200-208) Moreover, CD30 is also expressed on T cells, making CD30-specific CAR$^+$ T cells candidates for T-ALL therapy, but no T-ALL-specific CARs have been generated to date. Only one report of CARs targeting CML has been made thus far but the actual TAA was not examined.(209) CARs specific for CD33 and CD123 have been generated to target AML, but may have off-target effects due to the importance of CD33 and CD123 in hematopoiesis and viral immunity because of their expression on plasmacytoid dendritic cells that are critical producers of type-I interferons needed for viral clearance.(210-214) OvCa has been the target of multiple CARs including those specific for mesothelin, α-Folate Receptor (αFR), and folate-binding protein (FBP).(42, 215-219) Renal cell carcinoma has been targeted through the carbonic anhydrase IX (CAIX), which has minimal expression in normal tissues and is increased in hypoxia.(220-222) Carcinoembryonic antigen (CEA) is a developmental antigen absent on normal tissue and up-regulated in malignant cells, and CARs targeting CEA have been developed for pancreatic and colorectal cancers.(223, 224) Similarly, the oncofetal antigens h5T4 and ROR1 (discussed in **Chapter I.B.2**) are only expressed during development and CARs specific for these antigens can target multiple tumor types.(77, 199, 223) The differences between published ROR1-specific CAR T cells and the ones developed in this dissertation are discussed in detail in **Chapter II**.

Both CAR and mAb immunotherapies have had much success targeting human epidermal growth factor receptor-2 (EGFR2, HER2, or ERBB2), which is expressed highly in many cancers.(194, 225-228) However, there is low-level expression of HER2 on normal tissues, which caused an "on-target/off-target" toxicity in the only trial to date testing CAR$^+$ T cells specific for this TAA on breast cancer, thereby limiting its application.(229) Other EGFR members have been targeted with CARs, including EGFRvIII, which is uniquely expressed on glioblastoma.(230-232) Even glycoproteins (Lewis-Y antigen) can be targeted by CARs, and Lewis-Y antigens are typically studied in the context of EGFR family members.(233) The ganglioside GD2 and L1-cell adhesion molecule (L1-CAM) are common expressed on neuroblastoma, melanoma, and sarcoma (GD2 only), and CARs targeting these TAA were shown to control neuroblastoma growth.(234-239) In addition to GD2 and L1-CAM, high molecular weight melanoma-associated protein was used as a target for melanoma.(240) Melanoma is highly responsive to immunotherapy, and complete responses have been generated from a single infusion of tumor infiltrating lymphocytes (TILs).(241, 242) Prostate cancer has two specific antigens with limited expression outside of the prostate, prostate stem cell antigen (PSCA) and prostate-specific membrane antigen (PSMA), which were both targeted with CARs.(243-245) MUC1 was also another CAR target for both prostate and breast cancers.(246, 247) Other ubiquitous tumor markers, e.g. tumor associated glycoprotein-72 (TAG72) and epithelial glycoprotein-2 (EGP-2) have been targeted by CARs for multiple cancer therapies.(248, 249) Angiogenesis is even the target of a CAR via specificity for Vascular Endothelial Growth Factor Receptor 2 (VEGFR2), which is crucial for introducing new blood vessels into the tumors.(250,

251) However, there are major concerns of long-term persistence of these VEGFR2-specific cells in terms of regular vasculature growth. Lastly, receptors expressed on tumors can be targeted by "zetakines," which function like CARs but replace the scFv of the CAR with the ligand for a receptor of interest. For example, IL13-Receptor-$\alpha$-2 (IL13R$\alpha$2) was targeted by an IL13 fused to T cell signaling domains to target glioblastoma multiforme and neuroblastoma.(252-255) As outlined, many tumor antigens have been targeted by CARs, highlighting the enthusiasm given to this immunotherapy.

## I.D.3. Clinical Trials with CAR^{+} T cells

Many of the CARs described above have been translated into T cell immunotherapies for cancer patients and have resulted in promising objective clinical responses.(200, 241, 242, 256, 257) The majority of the trials have been focused on CARs developed from the FMC63 mAb specific for CD19.(186, 258, 259) CD19-specific CAR^{+} T cells have eliminated tumor from patients resulting in B cell aplasia, a litmus test for long-lived clinical responses.(4-7, 260) It was in this model that second generation CARs proved to have superior anti-leukemia effects compared to first generation CARs. Furthermore, long-lived persistence of CAR^{+} T cells has been achieved by rendering them bi-specific to TAA and Epstein-Barr virus (EBV)-specific antigens through skewing TCR repertoire in *ex vivo* co-cultures with EBV-transformed lymphoblastoid cell lines (LCL).(206, 211, 212, 236, 237) The most striking clinical responses, including maintained complete responses, have been achieved with second generation

CD19-specific CAR$^+$ T cells signaling through CD137 and CD3$\zeta$.(4, 7, 32) The exact reason why these cells out-performed other CARs signaling through CD28 and CD3$\zeta$ is unknown at present, and pre-clinical models have not shown many differences between CD28 and CD137 CARs.(5, 6) This is an active area of investigation and **Chapter II** focuses on this question directly with ROR1-specific CARs that are in the approval stages for a Phase I clinical trial. The focus of all Phase I clinical trials, of which most CAR trials have been, is safety and establishing a maximum tolerated dose. Unfortunately, there have been 2 deaths on CAR$^+$ T cell clinical trials. The first death followed administration of CD19-specific T cells to an elderly patient, who later died of complications not thought to be directly linked to the immunotherapy.(261) In contrast, the second death was directly attributed to the CAR$^+$ T cells. In this study, a third generation CAR (CD28, CD137, and CD3$\zeta$) specific for HER2 (based on the monoclonal antibody trastuzumab) was used to treat breast cancer, and following infusion of $10^{10}$ T cells, the patient died of cytokine storm in response to basal levels of HER2 on the lungs.(229) This tragedy has heightened the safety concerns around CAR$^+$ T cell immunotherapy, and TAA choice, CAR design, and T cell dose are being closely monitored in current and future trials.(262) Nonetheless, clinical trials are currently accruing with CAR$^+$ T cells targeting HER2 for sarcoma (NCT00902044), glioblastoma multiforme (NCT01109095), and multiple cancer (NCT00889954) treatments (http://www.clinicaltrials.gov/). Clinical trials with CAR-modified T cells specific for $\alpha$FR were not effective at treating advanced ovarian cancer, and the lack of efficacy was attributed to lack of persistence of T cells *in vivo*.(42) Other trials targeting solid tumors with TAA, e.g. GD2, L1-CAM, CAIX, and IL13R$\alpha$2, which are similar to

HER2 expression in that there is some expression on normal tissues, have been safe and sometimes effective at reducing tumor burden.(186, 220, 221, 235-237) Therefore, the safety and efficacy of a particular CAR$^+$ T cell clinical trial may vary from investigator to investigator due to nuance in a number of variables surrounding propagation and CAR design and/or from variability between individual patients.

## I.E. *Ex Vivo* Propagation of T cells

Many platforms exist for the propagation of T cells *ex vivo*, and this dissertation focuses on the use of *Sleeping Beauty* (SB) transposition for gene transfer into T cells followed by propagation on artificial antigen presenting cells (aAPC). This non-viral system for propagating T cells can be contrasted to viral-mediated gene transfer in that the latter requires previous expansion, e.g. with agonistic antibodies or stimulating beads, in order to transduce cells with the transgene of interest and the former does not require previous expansion but rather propagates the T cells *ex vivo* following gene transfer. The SB/aAPC strategy has been translated into the clinic, and modification of the current SB/aAPC will be used to streamline translation of therapies developed in this thesis to the clinic.

### I.E.1. *Sleeping Beauty* Transposition-mediated Gene Transfer

Non-viral gene transfer with SB transposition establishes stable transgene expression in human cells.(263, 264) SB genes are originally derived from fish that were undergoing

active transposition in their evolutionary maturation and were adapted for transposition into human cells.(265) In short, a DNA transposon with flanking inverted repeats and direct repeats is ligated into the human genome at TA dinucleotide repeats by the SB transposase enzyme.(266) TA dinucleotide repeats are randomly distributed in the human genome, yielding potential for random integration into the genome and has shown to be safe in regards to transgene insertion in pre-clinical studies.(267-269) This is of particular importance in gene therapy as inappropriate integration at gene start sites or promoters, within exons, or even distal to genes within enhancers or repressors can cause cellular transformation. Lentiviruses and γ-retroviruses have higher efficiency in transgene delivery than SB, but these vectors are known to integrate near genes or within genes.(186) Moreover, this was a particular problem in gene therapy trials treating X-linked severe combined immunodeficiency syndrome (X-SCID) where roughly half of the patients receiving transduced cells later developed leukemia as a result of integration near the *LMO2* gene.(270, 271) In contrast, no preference towards a particular chromosome or gene "hotspot" has been detected with SB.(267) Application of SB to human T cells has worked as a two DNA plasmid system, where one plasmid contains the SB transposon with the transgene of interest, e.g. CAR, and the other plasmid encodes the SB transposase.(272) Electro-transfer of the DNA plasmids by Amaxa nucleofection into quiescent peripheral blood mononuclear cells (PBMC) results in transient expression of SB transposase that then ligates the CAR transposon into the genome. As soon as the SB transposase mRNA is degraded translation of SB transposase protein is halted, thereby limiting the chances of additional transposition events. CAR expression can be encouraged through the co-culture of T cells on aAPC

that express cognate antigen for the CAR.(273) aAPC serve as feeder cells, and recursive stimulations with γ-irradiated aAPC promote CAR-specific growth. Typically, after 30 days of co-culture >90% of cells will express CAR (**Figure 5**). Thus, SB transposition is an efficient gene transfer modality in T cells and modified T cells can be expanded *ex vivo* by aAPC co-culture.
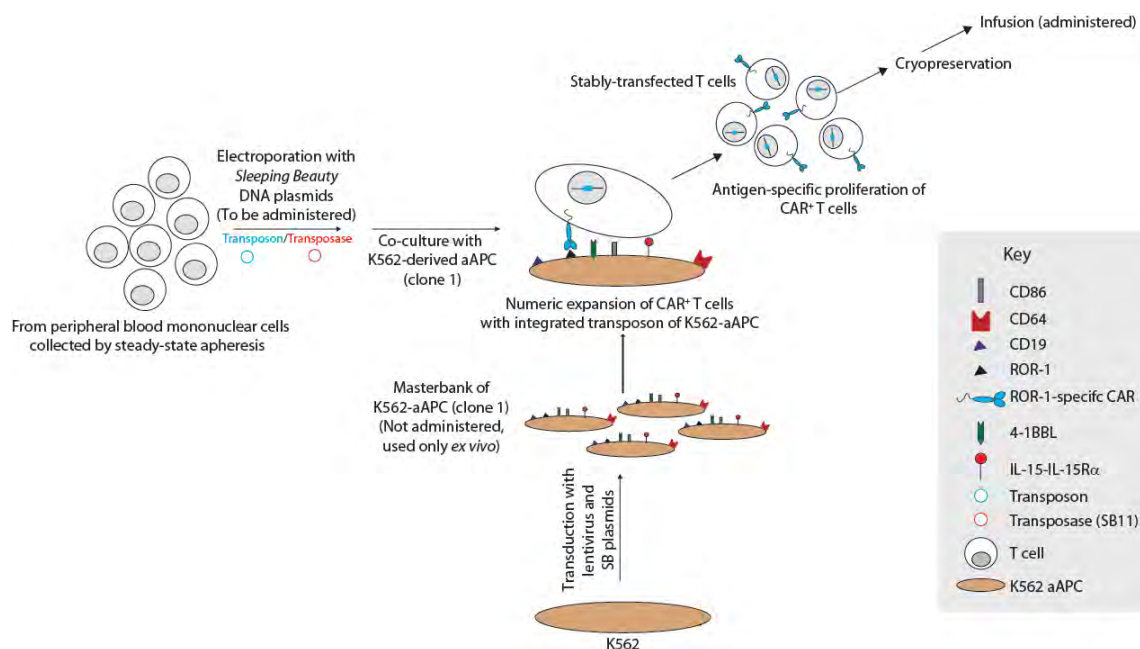
**Figure 5. Schematic of CAR<sup>+</sup> T cells Expansion on aAPC.** PBMC are isolated by Ficoll-Hypaque or steady state apheresis and are electroporated with plasmids encoding either (i) *Sleeping Beauty* transposase or (ii) *Sleeping Beauty* transposon containing CAR. Transient expression of CAR is observed the following day, and recursive stimulations with K562-derived aAPC are performed weekly with exogenous IL2 and/or IL-21. Pictured here are the clone#1 aAPC that expresses CD19, ROR1, CD64, CD86, CD137L, and IL15/IL15Rα. Following a month of co-culture on aAPC, stable CAR expression is achieved and clinically-relevant numbers of CAR<sup>+</sup> T cells are ready for cryopreservation and then infusion into cancer patients.

I.E.2 Artificial Antigen Presenting Cells

CARs stimulate T cells independent of their TCR specificity, and a primary aim of this propagation schema is to stimulate the CAR without affecting TCR repertoire by avoiding TCR/MHC interactions. Classical dendritic cells, thought of as "professional" APC, are infrequent in peripheral blood, laborious to manipulate, have limited replicative ability, and would need to be generated in the autologous setting for each immunotherapy patient. For these reasons, an alternative means for CAR-specific proliferation was sought after with the goal of serving as a global "off-the-shelf" bank of aAPCs to stimulate T cells independent of their MHC typing.

*I.E.2.a Unique Features of K562 for Antigen Presentation*

K562 has become an efficient aAPC line because it (i) lacks most MHC Class-I molecules, (ii) can be genetically modified easily, and (iii) proliferates robustly for easy cell banking and scale-up purposes.(273-276)  The lack of MHC Class-I molecules (no A or B but limited C) on the K562 surface is advantageous because CD8-specific allogeneic reactivity is minimized or could be tailored to certain HLA restriction for TCR-specific responses.(277, 278) Expansion of T cells on aAPC has shown that polyclonal TCR repertoire is readily achieved, suggesting that the aAPCs do not skew endogenous TCR-response to a particular affinity or antigen.(263) Another important characteristic of using K562-derived aAPC is their susceptibility to further gene modification by either non-viral or viral mediated gene transfer. For instance, a master aAPC cell bank was modified with both IL15 fusion protein to IL15 receptor-$\alpha$

(IL15/IL15Rα) and ROR1 antigen for memory formation and propagation of ROR1-specific T cells, respectively (**Chapter II**). Also, HLA-Cw3 was detected on K562 cells, so Cw3 was efficiently removed with zinc-finger nucleases to create HLA$^{-/-}$ K562 cells (Torikai H, Cooper LJN, and Lee DA, unpublished observations) in order to generate new aAPC completely devoid of HLA Class-I molecules. Thus, working cell banks can be easily re-tooled to ask biological questions regarding aAPC mechanics and/or maximize therapeutic cell output. Given the apparently unlimited proliferative capacity of K562 cells and their genetically modified counterparts, optimization of stimulations can be done easily and changed at will with options to use high ratios of aAPC to T cells. Furthermore, γ-irradiation of aAPC prior to co-culture with T cells is well tolerated by K562 in acute phases but eventually subjects the aAPC to death (typically 3 days) thereby eliminating most risk for unintended transfer of this tumor cell line into patients.(273) Therefore, K562 cells are an ideal source for antigen presentation and T cell stimulation.

*I.E.2.b. Established aAPC Cell Banks and Clinical Trials with aAPC*

As of now, four clinical trials have used K562-derived aAPC as T cell and NK cell expansion platforms at MD Anderson (NCT01653717, NCT01619761, NCT00968760, NCT01497184). Clone#4 aAPC generated at University of Pennslyvania (UPenn) was used successfully to expand CD19-specific CAR$^+$ T cells in both autologous and allogeneic settings.(57, 263, 272, 273, 279-281) The surface phenotype of clone#4 is characterized by expression of: (i) CD19, (ii) CD32 (as an endogenous protein), (iii)

CD64, (iv) CD86, (v) CD137L, and enhanced green fluorescence protein (eGFP; surrogate marker for IL15 expression). Similarly, clone#9 aAPC was also generated at UPenn and has a surface phenotype of: (i) CD19, (ii) CD32 (as an endogenous protein), (iii) CD64, (iv) CD86, and (v) CD137L. Clone#9 aAPC was further modified to express membrane-bound IL21 for trials propagating NK cells.(275) Translation of expansion protocols into the clinic was readily achieved and validated this approach. Patients treated with aAPC-expanded lymphocytes did not show toxicity, suggesting that this is a safe approach (Cooper LJN, unpublished observations). Thus, aAPC will be used for the propagation of T cells in this dissertation for direct clinical application.

**I.F. Dissertation Specific Aims**

This dissertation has three major specific aims, which attempt to solve the gaps in the above knowledge and/or application of immunotherapy. More specifically, these aims are directed at either harnessing the inherent anti-tumor immunity of T cells for cancer therapy, modifying T cells with natural anti-tumor capacity with CARs for enhanced specificity, or re-directing T cells with unpredictable anti-tumor immunity to cancer through CAR expression. This multivariate approach has resulted in approval of one Phase I clinical trial and holds the potential to result in other clinical trials for treatment of both solid and hematological tumors.

I.F.1 Specific Aim#1: To evaluate whether ROR1-specific T cells can target ROR1$^+$ tumor cells while sparing normal tissues. The *hypothesis* of this specific aim is that

ROR1-specific CARs will re-direct the specificity of T cells to target ROR1$^+$ malignancies and that CARs signaling through CD137 will be superior to CD28 in therapeutic efficacy. The *rationale* for this specific aim is that (i) ROR1 is a candidate TAA because it is expressed on a number of tumors but is not on most normal tissues, (ii) the 4A5 monoclonal antibody specific for ROR1 can be adapted to generate a CAR, (iii) CARs can re-direct T cells to TAA and empower them to kill TAA$^+$ malignancies, and (iv) cancer patients treated with CAR$^+$ T cells have achieved complete responses. *Sub-Aim 1.1. To generate ROR1-specific CAR$^+$ T cells.* Sequences from 4A5 antibody hybridoma will be constructed into second generation ROR1-specific CARs signaling through (i) CD28 and CD3ζ (ROR1RCD28) or (ii) CD137 and CD3ζ (ROR1RCD137), which will be part of SB transposons for stable CAR expression in T cells. CAR$^+$ T cells will be propagated on γ-irradiated ROR1$^+$ aAPC (clone#1), and CAR$^+$ T-cell numeric expansion will be monitored by inferred cell counts and flow cytometry for 28 days. *Sub-Aim 1.2. To phenotype ROR1-specific CAR$^+$ T cells.* Extended phenotyping for memory and homing markers will be performed by flow cytometry at the end of the co-culture period. Genotyping will also be performed with nCounter gene expression platform for TCR isotype expression and lymphocyte-associated genes. *Sub-Aim 1.3. To assess whether CAR$^+$ T cell function is specific for ROR1.* Cytokine production and 4-hour chromium release assay (CRA) will be used to evaluate CAR$^+$ specificity in responding to ROR1$^+$ targets with ROR1$^{neg}$ targets as negative controls. ROR1$^+$ leukemia xenografts will be established in immunocompromised mice which will be treated with CAR$^+$ T cells to evaluate tumor clearance *in vivo*.

I.F.2. Specific Aim#2: To assess whether a CD19-specific CAR expressed on γδ T cells will render them bi-specific to tumors through their TCR and CAR. The *hypothesis* of this specific aim is that enforced CAR expression on γδ T cells would stimulate them independent of their TCRγδ, thus leading to expansion of γδ T cells with polyclonal TCRγδ repertoire, and would amplify the anti-tumor effects from TCRγδ towards TAA+ malignancies through the CAR. The *rationale* for this specific aim is that (i) γδ T cells have inherent anti-tumor immunity through a number of combinations of TCRγ and TCRδ pairings, (ii) the use of γδ T cells in the clinic is currently restricted to Vγ9Vδ2 even though other γδ T cell lineages have anti-tumor reactivity, (iii) CARs stimulate T cells independent of their TCR, (iv) electroporation of SB transposons containing the CAR can be achieved in quiescent PBMC with a polyclonal repertoire of γδ T cells, and (v) CD19-specific CAR transposon plasmids and CD19+ aAPC are currently in clinical trials at MD Anderson and these reagents can be used to quickly translate findings from this specific aim into clinical trials. *Sub-Aim 2.1. To propagate CAR+ γδ T cells on aAPC.* The second generation CD19-specific CAR (CD19RCD28) currently in clinical trials is available as highly pure DNA and will be used for gene transfer into quiescent PBMC from which CAR+ γδ T cells will be propagated on CD19+ aAPC. CAR expression and inferred cell counts will be used to evaluate CAR+ γδ T cell numeric expansion. *Sub-Aim 2.2. To phenotype CAR+ γδ T cells.* After a month of expansion on aAPC, CAR+ γδ T cell surface phenotypes will be evaluated for T cell and memory molecules by flow cytometry and TCRγδ allele expression will be assessed by nCounter gene expression analysis. *Sub-Aim 2.3. To determine the ability of CAR+ γδ T cells to*

*functionally respond to tumors.* Cytokine production and 4-hour CRA assays will be tested against CD19$^+$ tumor targets with CD19$^{neg}$ targets serving as negative controls. Autologous CAR$^{neg}$ γδ T cells will be used to compare CAR-specific responses to CD19$^+$ tumors. CD19$^+$ leukemia xenografts will be established in immunocompromised mice which will be treated with CAR$^+$ γδ T cells to evaluate anti-tumor effects *in vivo*.

I.F.3. Specific Aim#3: To evaluate the inherent anti-tumor activity of aAPC-expanded γδ T cells against solid and hematological cancers. The *hypothesis* of this specific aim is that aAPC will expand polyclonal γδ T cells that will have broad anti-tumor immunity. The *rationale* for this specific aim is that (i) CAR$^{neg}$ polyclonal γδ T cells proliferated in parallel to CAR$^+$ γδ T cells described in specific aim#2 on aAPC, (ii) no current expansion protocols exist for polyclonal γδ T cells for the clinic, (iii) aAPC are currently in clinical trials and are available as a master cell bank in the manufacturing facility at MD Anderson, (iv) γδ T cells expressing Vδ1 are correlated with long-term remissions in cancer therapy but have not been directly infused as an adoptive immunotherapy, (v) γδ T cells expressing Vδ2 have shown anti-tumor effects as direct adoptive immunotherapies, (vi) γδ T cells expressing Vδ3 have not been described to have direct anti-tumor immunity leaving a gap in the field of knowledge, and (vii) a polyclonal approach to γδ T cell immunotherapy could target multiple ligands on the tumor through a diverse repertoire of TCRγδ. *Sub-Aim 3.1. To propagate γδ T cells on aAPC.* PBMC or UCB will be sorted for γδ T cells, and then co-cultured with aAPC used in clinical trials at MD Anderson. Flow cytometry and inferred cell counts will be

used to evaluate proliferation of γδ T cells. Subsets of γδ T cells will also be sorted and expanded as co-cultures with clinical aAPC to assess differences in γδ T cell lineages. *Sub-Aim 3.2. To phenotype γδ T cells expanded on aAPC.* After one month of co-culture on aAPC, the surfaces of polyclonal or sorted γδ T cells will be evaluated for T cell and memory markers by flow cytometry and TCR allele expression will be assessed on nCounter gene expression platform. *Sub-Aim 3.3. To examine the range of killing capabilities by aAPC-expanded γδ T cells.* Polyclonal or sorted γδ T cells will be evaluated for their ability to produce cytokines in response to TCR stimulation or co-culture with tumor cells derived from solid and hematological cancers. Standard 4-hour CRA will be used to assess acute cytolysis and long-term co-cultures will evaluate durable killing abilities. Neutralizing antibodies will be employed to determine the specificity of killing. OvCa xenografts will be established in immunocompromised mice which will be treated with polyclonal γδ T cells to test their tumor clearance *in vivo*.

# CHAPTER II

## Clinical Implications for ROR1-specific T cells

### II.A. Hypothesis and Rationale

The *hypothesis* of this chapter is that ROR1-specific CARs will re-direct the specificity of T cells to target ROR1$^+$ malignances and that CARs signaling through CD137 will be superior to those signaling through CD28 in therapeutic efficacy. The *rationale* for this chapter is that (i) ROR1 is a candidate TAA because it is expressed on a number of tumors but not on most normal tissues, (ii) the 4A5 monoclonal antibody specific for ROR1 can be adapted to generate a CAR, (iii) CARs can re-direct T cells to TAA and empower them to kill TAA$^+$ malignances, and (iv) cancer patients treated with CAR$^+$ T cells have achieved complete responses. This chapter describes pre-clinical testing of ROR1-specific T cells that have clinical implications as cancer immunotherapies.

### II.B. Introduction

Current clinical trials use T cells expressing CARs specific for CD19, an antigen expressed on the surfaces of all B cells, to eliminate refractory B-cell malignancies.[4, 57, 184, 186] However, there is also loss of normal CD19$^+$ B cells in patients undergoing this therapy, which can result in serious health complications including loss of humoral immunity.[7, 32] Furthermore, loss of CD19$^+$ B cells in an elderly patient

treated with CD19-specific CAR⁺ T cells resulted in death from an opportunist viral infection.(261) ROR1 is absent on most normal B cells and other healthy tissues (**Chapter I.B.2.**), but is expressed on many B-cell tumors (mantle cell lymphoma (MCL), ALL with t(1:19) translocations, and >95% of CLL) and solid tumors (lung and breast cancer, OvCa, PaCa, renal cell carcinoma, and melanoma) where ROR1 expression is required for cellular growth and survival.(13, 64, 66, 67, 75, 79, 80, 282) Thus, CARs targeting ROR1 instead of CD19 would allow for tumor elimination while sustaining the normal B cell repertoire, and ROR1-specific T cells have the potential for use in a number of solid tumors.

The design of the CAR is a source of debate at present. Striking clinical data, including complete responses, were observed in ALL and CLL patients treated with second generation CD19-specific CARs having CD137 (41BB) endodomain or the more frequently used CD28 region.(5-7, 32) However, the differences between the two CARs or their mechanisms of improved efficacy over other CAR clinical trials are unknown at present. CAR clinical trials targeting CD19 open at MD Anderson use the CD28 moiety (NCT01653717, NCT00968760, NCT01497184), but are being adapted to (i) directly compare CD28 to CD137 CARs and/or (ii) replace CD28 CARs with CD137 CARs. These trials, and those performed at other independent centers, will aim to validate these remarkable responses and determine whether CD28 or CD137 is the ideal co-stimulatory domain for CD19-specific CARs.

However, these results may not necessarily hold true for targeting different antigens due to differences in antibody affinity and/or antigen expression. Direct immunotherapy of ROR1-specific antibody (through clone 2A2) has been proposed as

an option for leukemia and broader cancer treatment, but this antibody appears to have strong cytoplasmic staining in a number of normal tissues (despite absence of ROR1 mRNA expressed in these tissues) and directly binds to adipocytes that express small amounts of ROR1 mRNA.(77, 81, 283) CARs have been developed from the 2A2 (mouse) and R12 (goat) antibodies, and CAR$^+$ T cells were generated in central memory T cells (T$_{CM}$) that could then efficiently lyse ROR1$^+$ tumor, but their reactivity towards normal tissues outside of normal B cells was not evaluated.(77, 199) The optimal 2A2 and R12 CARs for expression in T$_{CM}$ cells had short extracellular domains (14 amino acids) with CD137 and CD3ζ signaling endodomains. In contrast to other ROR1-specific antibodies, the 4A5 clone developed by Dr. Thomas J Kipps (Moores Cancer Center, UCSD) has not been shown to bind any normal tissues, except hematogones (dispensable B-cell precursors), but is highly reactive to a number of cancers, including leukemia, OvCa, and PaCa.(66, 67, 75, 79) Therefore, this clone was chosen for generation of ROR1-specific T cells in the expansion system developed at MD Anderson that has a number of differences to the previous studies, including (i) 4A5 antibody specificity, (ii) expression of CAR in polyclonal peripheral T cells containing naïve and T$_{CM}$ reported to have maximal efficacy as CAR$^+$ T cells,(131) (iii) propagation of CAR$^+$ T cells on aAPC containing membrane-bound IL15/IL15Rα fusion protein for optimal cytokine signaling potency and memory formation, and (iv) expansion schema without the need for sorting steps that can complicate clinical translation. Thus, CARs developed based on this strategy are hypothesized to have efficient killing of ROR1$^+$ malignancies and could answer some of the same fundamental CAR questions in a broader set of peripheral T cells.

Clinical trials have not yet tested ROR1-specific CARs in humans, so this report of pre-clinical testing of ROR1-specific CARs aims to directly test CD28 and CD137 signaling CARs to streamline trial design and clinical efficacy for cancer treatments. "First-in-man" clinical trials open at MD Anderson translated (i) co-electro-transfer of CD19-specific CAR *Sleeping Beauty* (SB) transposon with SB transposase and (ii) expansion of CD19-specific CAR$^+$ T cell on CD19$^+$ aAPC into clinical manufacturing and were successfully transplanted into leukemia patients without toxicity or adverse event, suggesting that this is an effective and safe strategy (Cooper LJN, unpublished observation). This study builds upon these successes and adapts current (i) CAR plasmids, (ii) working aAPC cell banks expressing co-stimulatory molecules for endogenous co-stimulation of CD28 and CD137, and (iii) protocols for direct clinical application. A phase I clinical trial has been approved by the National Institutes of Health (NIH) DNA Recombinant Advisory Committee (RAC) based on the data herein and is currently under review at the MD Anderson Cancer Center Institutional Review Board (IRB). Thus, ROR1-specific CARs are close to being tested for the first time in cancer immunotherapy.

## II.C. Results

### II.C.1. ROR1 Surface Expression on Tumor Cells

Surface expression of ROR1 was detected on a number of leukemia cell lines, OvCa cell lines, and primary leukemia patient samples before proceeding with generating ROR1-specific CARs. The 4A5 monoclonal antibody has been shown to have high

49

affinity binding to ROR1,(75) and it was provided by Dr. Thomas J Kipps (UCSD) for testing ROR1 expression at MDACC. EL4 is a murine T-cell lymphoma cell line with low cross-reactivity with human T cells most likely due to their differences in MHC molecules. This cell line does not express human ROR1, thus they were genetically modified to express ROR1 in order to assess CAR-specific responses independent of their TCR interaction with MHC (**Figure 6a**). Human B-cell ALL cell lines were readily accessible and were profiled for ROR1 expression. As expected, ROR1 was present on some, but not all, B-ALL cell lines. More specifically, NALM6 and Kasumi2 tested negative and positive for ROR1, respectively (**Figure 6b**). ROR1 was also expressed on most (11 of 12) OvCa cell lines tested, which are best exemplified by ROR1$^+$ EFO27 cells and the only ROR1$^{neg}$ OvCa cell line tested, A2780 (**Figure 6c**). ROR1 was originally described as a cancer antigen in B-cell CLL, so primary B-cell CLL patient samples were acquired for testing in parallel with LCL derived from healthy donor B cells immortalized with EBV. Indeed, CLL samples stained for ROR1 while LCL did not (**Figure 6d**). These results corroborated the previous literature and gave us confidence to go forward with generating a ROR1-specific CAR designed from the 4A5 antibody.
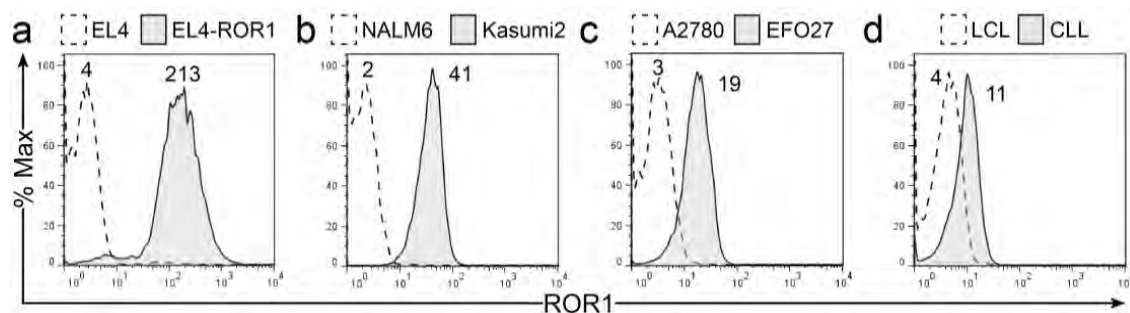
**Figure 6. Surface Expression of ROR1 on Tumors.** The 4A5 mAb specific for ROR1 was used to assess ROR1 expression on the surface of (**a**) EL4 parental (ROR1^{neg}) and genetically modified EL4-ROR1 cells, (**b**) B-ALL cell lines NALM6 and Kasumi2, (**c**) OvCa cell lines A2780 and EFO27, and (**d**) primary patient B-CLL cells or healthy donor LCL by flow cytometry. Mean fluorescence intensities (MFI) are displayed near corresponding histograms and legends are displayed above corresponding graphs.

II.C.2. ROR1-specific CAR Plasmid Construction

Two SB transposons were constructed with second generation ROR1-specific CARs for side-by-side comparison between the CD28 (ROR1RCD28) and CD137 (ROR1RCD137) endodomains (**Figure 7a and 7b**, respectively). CD19 constructs were prepared in parallel with the CD28 (CD19RCD28) and CD137 (CD19RCD137) endodomains as controls for current standard T cell therapy and were identical to ROR1-specific CARs except in two pieces. First, the single chain variable fragment (scFv) differ between the CD19 and ROR1 constructs where the FMC63 and 4A5 monoclonal antibodies specific for CD19 and ROR1 were used, respectively. Second, CD19 CARs use the colony-stimulating factor-2 receptor (CSF2R) signal peptide whereas ROR1 CARs use the murine IgGκ signal peptide. Human elongation factor-1α promoter was used to drive CAR expression of all CARs. Following the promoter, the CAR open reading frame was composed of (from 5' to 3'): signal peptide, scFv with Whitlow linker, modified extracellular IgG4-Fc stalk,(272) CD28 transmembrane domain, CD28 or CD137 endodomains, and intracellular CD3ζ containing three ITAM domains. Interspaced between the STOP codons and the polyadenylation (polyA) tail were unique oligonucleotides to distinguish the two CAR transposons by polymerase chain reaction (PCR). The CD28 constructs could be distinguished from CD137 constructs by the "SIM" and "FRA" oligonucleotides, respectively. Thus, detection of T cell persistence in patients undergoing ROR1-CAR T cell therapy can be monitored and can corroborate flow cytometry data. SB indirect repeats flanking the promoter (5' end) and the polyA tail (3' end) defined the CAR transposons to be integrated within TA repeats in the human T cell genome. Lastly, kanamycin resistance was used to

selectively amplify CAR plasmids in bacteria to large quantities (0.5 – 1.0 mg), which were cleared for transfection after testing negative for endotoxin. In summary, these two ROR1-specific CAR plasmids mimic current plasmids used for CD19-specific CAR clinical trials at MD Anderson and should be directly translatable to the clinical setting.
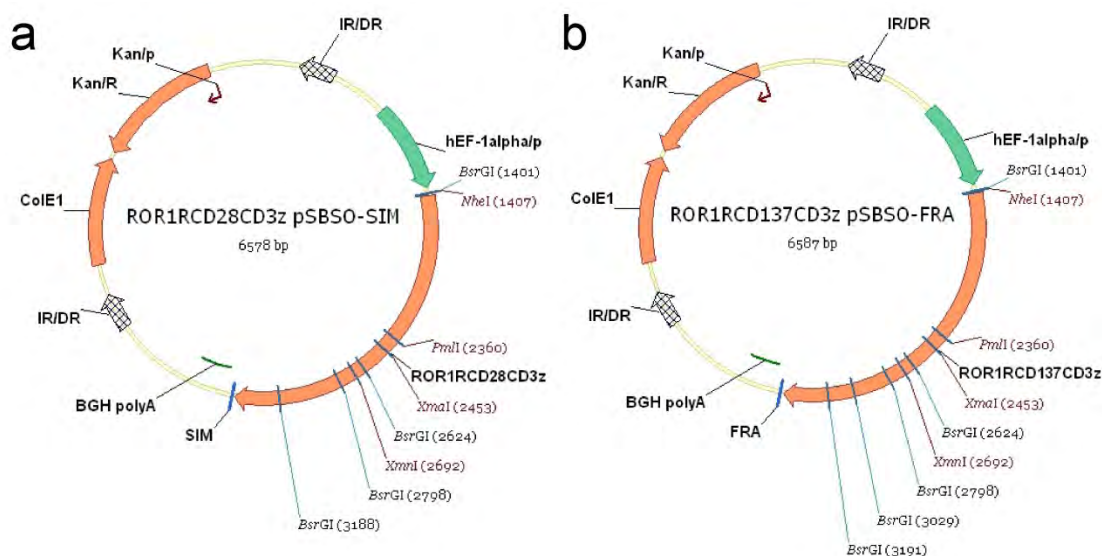
**Figure 7. ROR1-specific CAR Transposons.** DNA plasmid vector maps for (**a**) ROR1RCD28 and (**b**) ROR1RCD137. Abbreviations are as follows, IR/DR: *Sleeping Beauty* Inverted Repeat, hEF-1alpha/p: Human Elongation Factor-1 alpha region hybrid promoter, ROR1RCD28CD3z: Human codon optimized ROR1-specific scFvFc:CD28zeta chimeric antigen receptor, ROR1RCD137CD3z: Human codon optimized ROR1-specific scFvFc:CD137zeta chimeric antigen receptor, SIM: "SIM" PCR tracking oligonucleotides, FRA: "FRA" PCR tracking oligonucleotides, BGH polyA; bovine growth hormone polyadenylation sequence, ColE1: A minimal *E.coli* origin of replication, Kanamycin (Kan/R): Bacterial selection gene encoding Kanamycin resistance, Kanamycin promoter (Kan/p); Prokaryotic promoter. Digestion with BsrGI enzyme can distinguish the two plasmids, which have high degrees of similarity. The entire plasmid sequences were verified by Sanger-based sequencing techniques.

## II.C.3. Development of ROR1$^+$ aAPC (clone#1)

aAPC have been shown to propagate T cells *ex vivo* through (i) expression of cognate antigen or (ii) activation through membrane-bound antibody. However, current clinical K562-based aAPC cell banks at MD Anderson do not express ROR1. Therefore, a new aAPC was developed to express ROR1 and an IL15 fusion protein to the IL15 receptor-$\alpha$ (IL15/IL15R$\alpha$) along with the other molecules present on aAPC surfaces. Trans-presentation of IL15 by IL15R$\alpha$ has been shown to have higher signaling potency than IL15 alone in other models.(284, 285) Clone#1 feeder cells were derived from the K562 cell line, which was previously made to express CD19 antigen, co-stimulatory molecules (CD86 and CD137L), and Fc receptors (endogenous CD32 and introduced CD64) for loading of agonistic anti-CD3 antibody (OKT3). Thus, the CAR$^+$ T cells had the potential to receive co-stimulation through the CAR and from endogenous binding of CD28 and CD137 on the T cell to CD86 and CD137L, respectively, on the aAPC. Prior to co-culture, aAPC were γ-irradiated (100 Gy) and typically die within 3 days of co-culture. Clone#1 aAPC were phenotyped prior to co-culture to ensure that all markers were present at >80% (**Figure 8 right panels**). Negative and positive controls were parental K562 cells (**Figure 8 left panels**) and clone#4 aAPC (**Figure 8 middle panels**) used in CD19-specific CAR$^+$ T cell clinical trials at MD Anderson, respectively. The expression of IL15 by clone#4 is detected with eGFP as a surrogate marker but IL15 was directly detected on the surface of the clone#1 cells. Cytokine support, co-stimulation, and antigen expression by clone#1 aAPC gave us confidence in its ability for use in CAR$^+$ T cell propagation.
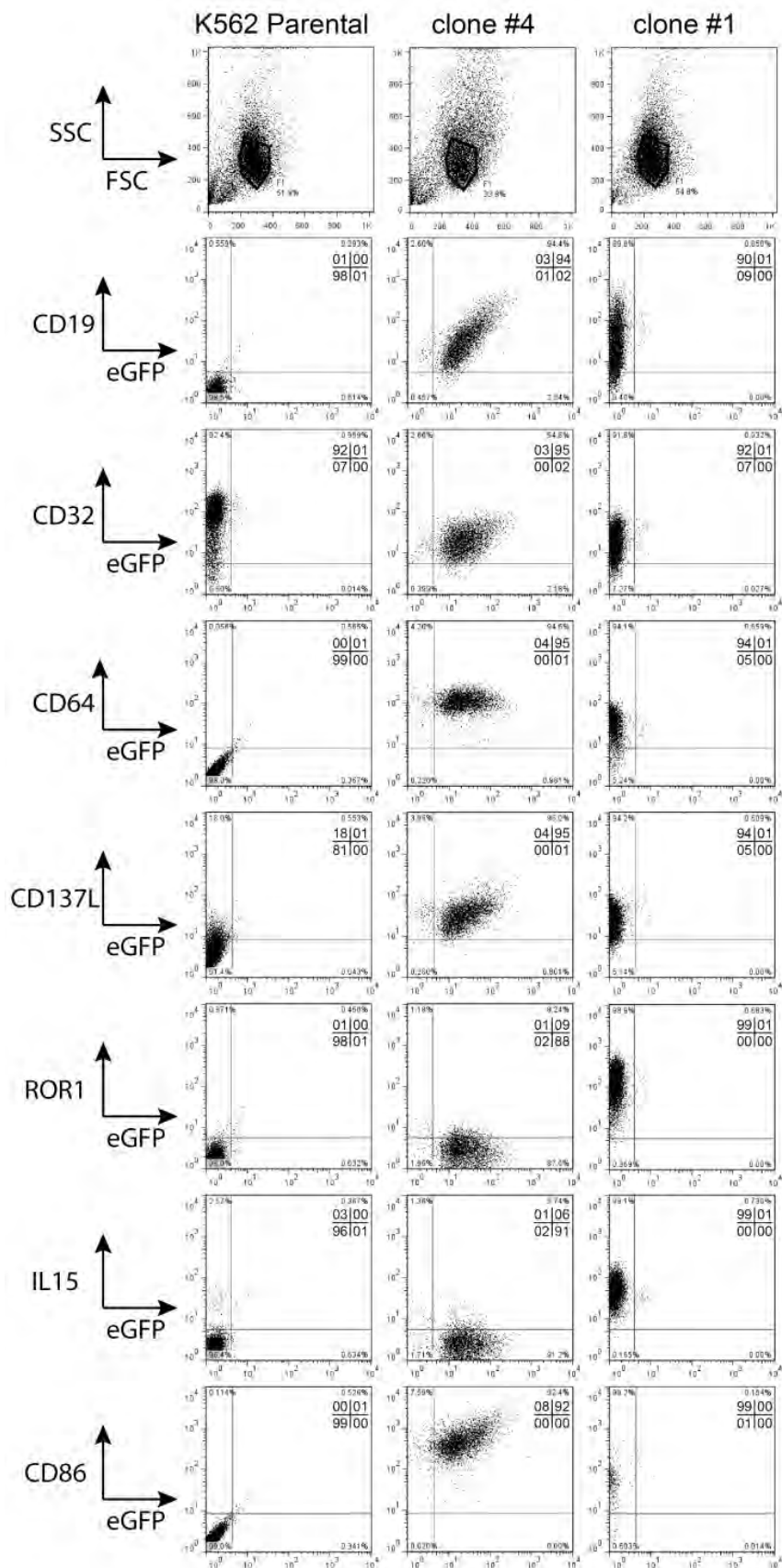
**Figure 8. Surface Phenotype of Clone#1 aAPC Used for ROR1-specific T cell Expansion.** Parental K562 (left), clone#4 aAPC (middle), and clone#1 aAPC (right) were stained for surface marker expression and were analyzed by flow cytometry. Top plots are forward scatter (FSC; x-axes) by side scatter (SSC; y-axes). Other plots were eGFP (x-axes) with the following on the y-axes from top to bottom: CD19, CD32, CD64, CD137L, ROR1, IL15, and CD86. Quadrant frequencies are displayed in the upper right corners.

II.C.4. CAR$^+$ T-cell Expansion on Clone#1 aAPC

Healthy donor PBMC were electroporated with (i) no DNA as a negative control for CAR expression, (ii) SB11 transposase and ROR1RCD28 transposon plasmids, or (iii) SB11 transposase and ROR1RCD137 transposon plasmids. The following day, cells were phenotyped for CAR expression on their surfaces where "no DNA" and isotype antibodies served as negative controls. Transient expression of CAR was detected in T cells at 41% ± 6% and 41% ± 8% (mean ± SD; n=3) for ROR1RCD28 and ROR1RCD137, respectively, as evidenced by co-staining for Fc (IgG4-Fc extracellular stalk of CAR) and CD3 (**Figure 9a**). Co-cultures were then initiated with γ-irradiated clone#1 aAPC and CAR$^+$ T cells at a 1:1 ratio. Similarly, γ-irradiated OKT3-loaded clone#4 aAPC and "no DNA" T cells were co-cultured at 1:1 ratio of total cells. Co-cultures were supplemented with IL21 (30 ng/mL) at the outset of co-culture and every 2-3 days thereafter. Recursive stimulations were performed every 7 days as above for four total stimulations, except that (i) IL2 (50 U/mL) was supplemented with IL21 starting at the second stimulation and (ii) NK cells were depleted from cultures with CD56 microbeads at day 15. At day 29, stable CAR expression was observed suggesting that clone#1 aAPC enforced CAR expression in T cells (**Figure 9b**). More specifically, CAR was expressed in T cells at 90% ± 3% and 79% ± 11% (mean ± SD; n=3) for ROR1RCD28 and ROR1RCD137, respectively, at the end of the co-culture. There was a difference between the transient and stable populations for ROR1RCD28 (p = 0.006) and ROR1RCD137 (p = 0.009), but the populations did not have significant differences in CAR expression (p = 0.184) following expansion. ROR1RCD137 had consistently lower mean fluorescence intensity (MFI) compared to ROR1RCD28 (51 ±

8 vs 102 ± 68, respectively) after expansion, but the reason for this is unknown at present. Recombinant rROR1 (rROR1; soluble extracellular domain) was purified and directly conjugated to a fluorescent marker (courtesy of Dr. Thomas J Kipps, USCD) for detection of antigen binding by CAR⁺ T cells. CD19-specific CAR⁺ T cells were expanded in parallel to serve as negative controls for rROR1 binding (**Figure 10a**). The CD19RCD28 had higher CAR expression than did ROR1RCD28, which could be explained by the differences in signal peptides used (human CSF2R and murine IgGκ, respectively). Nonetheless, ROR1RCD28 bound to rROR1, but CD19RCD28 and CAR$^{neg}$ T cells did not bind to rROR1 (**Figure 10b**). Proliferation kinetics between the two ROR1 CAR populations was similar in total cells counts ($p = 0.66$; Two-way ANOVA) and in CAR⁺ T cell counts ($p = 0.74$). Total cell proliferation closely coincided with CAR⁺ T cell proliferation kinetics for both ROR1RCD28 and ROR1RCD137 (**Figure 11**). ROR1RCD28 resulted in an average of $2.5 \times 10^9$ total inferred cell counts (range $1.4 \times 10^9$ – $4.0 \times 10^9$) and $2.2 \times 10^9$ CAR⁺ T cells (range $1.3 \times 10^9$ – $3.6 \times 10^9$), and ROR1RCD137 resulted in an average of $3.6 \times 10^9$ total inferred cell counts (range $3.7 \times 10^9$ – $8.2 \times 10^9$) and $2.9 \times 10^9$ CAR⁺ T cells (range $2.4 \times 10^9$ – $6.7 \times 10^9$). Thus, SB transposition resulted in stable CAR expression and co-culture on clone#1 aAPC led to clinically-relevant numbers of ROR1-specific T cells.
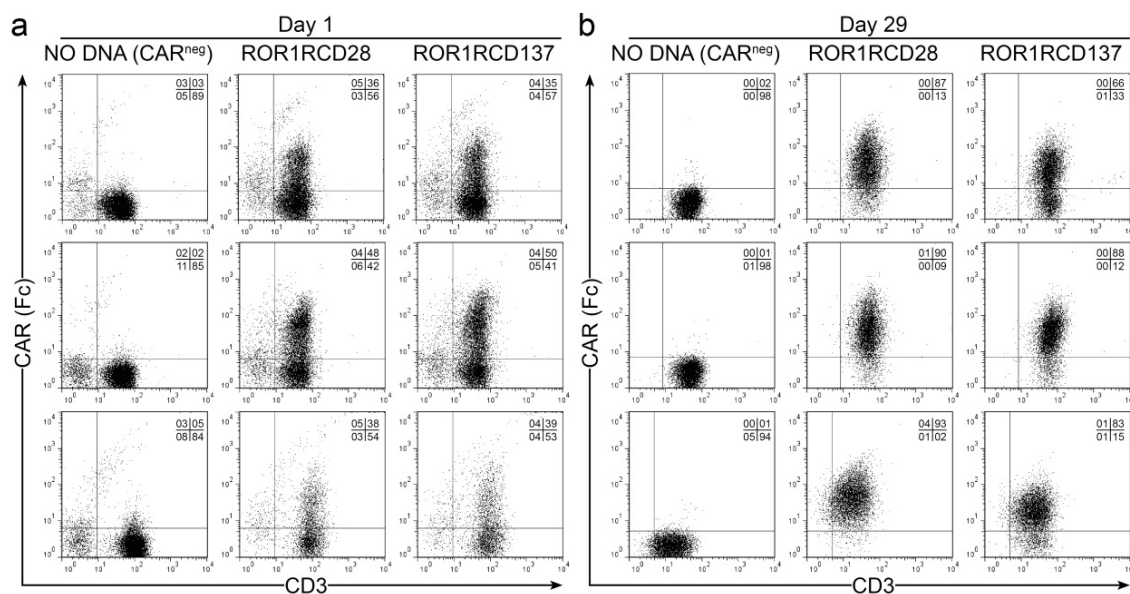
**Figure 9. CAR Expression in T cells Before and After Expansion on Clone#1 aAPC.** (**a**) Transient expression of ROR1RCD28 (middle) and ROR1RCD137 (right) T cells the day following electroporation where "no DNA" T cells (left) were used as negative controls. (**b**) Stable CAR expression in ROR1RCD28 (middle) and ROR1RCD137 (right) populations. T cells were marked by CD3 staining and CAR+ cells were detected with anti-Fc antibody. Quadrant frequencies are displayed in upper right corners.
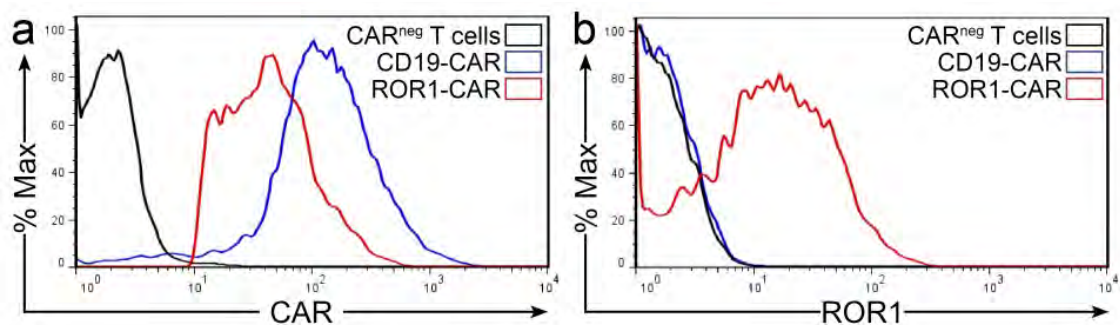
**Figure 10. rROR1 Antigen Binding by ROR1-specific T cells.** Recombinant ROR1 (rROR1) was purified and conjugated to fluorescent tag for detection of ROR1-specific T cells (ROR1RCD28). CD19-specific CAR$^+$ T cells (CD19RCD28) and "no DNA" CAR$^{neg}$ T cells were used as negative controls. (**a**) Fc detection of CARs and (**b**) rROR1 binding.
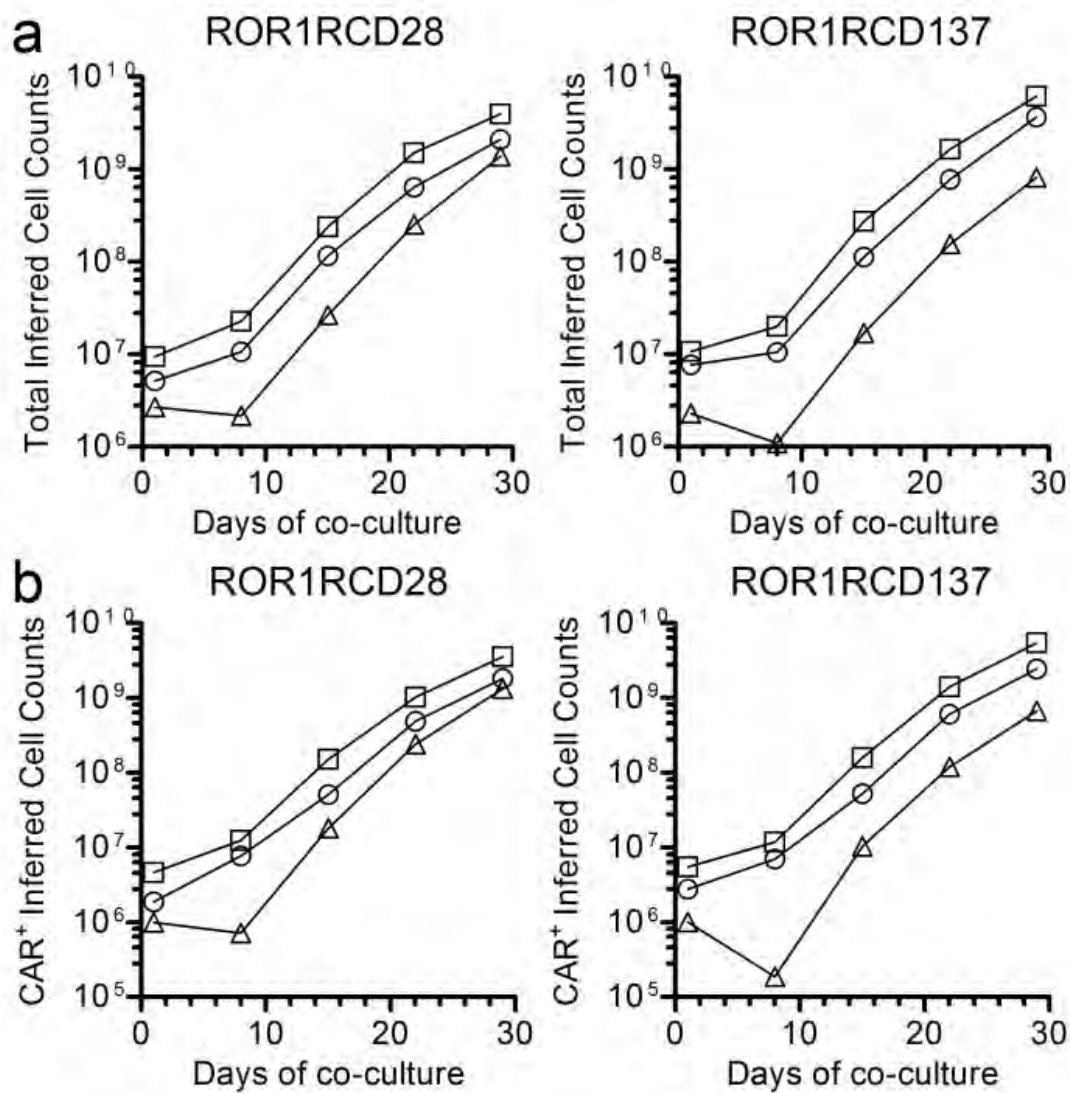
**Figure 11. Sustained Proliferation of CAR+ T cells.** (**a**) Total cells and (**b**) CAR+ T cell proliferation on clone#1 aAPC. ROR1RCD28 represented on the left and ROR1RCD137 shown on the right. Each symbol represents a different healthy donor.

II.C.5. Immunophenotype of ROR1-specific T cells

*II.C.5.a. T cell Immunophenotype of ROR1RCD28 and ROR1RCD137*

Following 29 days of expansion on irradiated clone#1 aAPC, ROR1RCD28 and ROR1RCD137 cells were profiled for (i) gene expression using the nCounter gene expression array platform (NanoString) and (ii) T cell surface proteins and memory markers by flow cytometry. A unique panel of lymphocyte genes was assembled for analysis on the nCounter and was termed "Lymphocyte CodeSet Array" or LCA (**Appendix A**). As expected, both δ and ε isoforms of CD3 (CD3D and CD3E, respectively) were highly expressed by both CAR$^+$ T cell populations, and there was higher expression of both CD3D and CD3E in ROR1RCD28 cells (**Figure 12a**). Expression of CD3ζ was not evaluated at the mRNA level because it could not be distinguished from CD3ζ on CAR intracellular domains. Nonetheless, >97% of CAR$^+$ T cells were CD3$^+$ on the cell surface (**Figure 12b**). There was also a trend of decreased expression of CD4 and CD8A transcripts in ROR1RCD137 cells relative to ROR1RCD28 and there was ~100 times more CD8A transcript than CD4 (**Figure 12a middle panels**). The same was observed at the protein level where both CARs preferentially expanded CD8$^+$ T cells over CD4$^+$ T cells and on average there were fewer CD4 and CD8 T cells in the ROR1RCD137 culture (**Figure 12b top panels and 12c**). This phenomenon of fewer CD4$^+$ and CD8$^+$ T cells is most likely attributed to small frequencies of γδ T cells (identified by CD3$^+$TCRγδ$^+$) that were present in the ROR1RCD137 cultures and not in the ROR1RCD28 cultures (**Figure 12b bottom panels**), because γδ T cells are commonly negative for both CD4 and CD8 but express

CD3.(286) Indeed, γδ T cells can proliferate on aAPC (**Chapters III and IV**), which suggests that they may compete for clone#1 for proliferative signal and diminish ROR1RCD137 cells from reaching >90% CAR$^+$ T cells. NK cells were present in cultures at Day 15 and were depleted with CD56 microbeads from all cultures, so negligible quantities of CD3$^{neg}$CD56$^+$ NK cells were detected at the end of the co-culture period two weeks later (**Figure 12b, middle panels**). CD56 was also expressed by T cells at the end of the co-culture period and is associated with MHC-unrestricted cytolysis (**Figure 12a and 12b**).(287) Significant differences between T cell surface protein expression were not observed (p = 0.322) between the two CARs in respect of CD3, CD4, CD8, CD56, NK cells, or γδ T cells (**Figure 12c**). These results suggest that CAR$^+$ T cells have canonical T cell phenotype features and on the basis of these evaluated markers were highly similar.
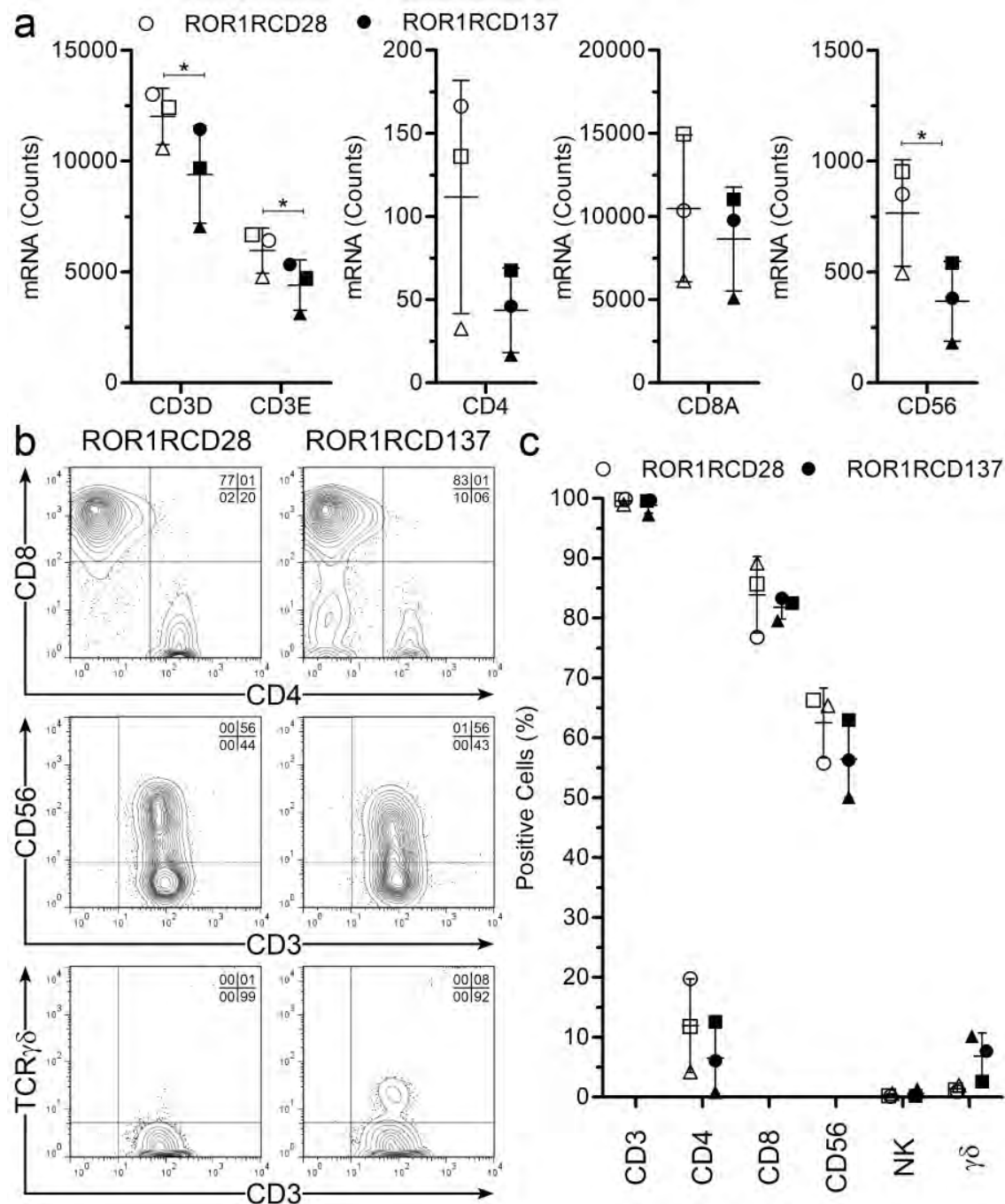
**Figure 12. Basic Immunophenotype of CAR^{+} T cells.** After 29 days of expansion on clone#1 aAPC, ROR1RCD28 and ROR1RCD137 cells were (i) lysed for mRNA expression analysis or (ii) phenotyped for T cell surface markers by flow cytometry. (**a**) RNA lysates were interrogated on nCounter gene expression array with "lymphocyte CodeSet array" (LCA) and normalized CD3 (far left), CD4 (middle left), CD8A (middle right), and CD56 (far right). mRNA expression are displayed for ROR1RCD28 (open shapes) and ROR1RCD137 (closed shapes). Student's paired, 2-tailed t-test was used

for statistical analysis (n = 3). *p<0.05 (**b**) CD4 (x-axes) and CD8 (y-axes) expression (top panels), CD3 (x-axes) and CD56 (y-axes) expression (middle panels), and CD3 (x-axes) and TCRγδ (y-axes) expression (bottom panels) of one of 3 representative donors. Gate frequencies are in the upper right corners and correspond to gate quadrants. (**c**) Frequencies of cells staining positive for each lymphocyte marker where each shape represents an individual donor, ROR1RCD28 are in open shapes and ROR1RCD137 are in closed shapes, NK cells were defined as $CD3^{neg}CD56^{+}$, γδ T cells were defined as $CD3^{+}TCR\gamma\delta^{+}$, and data are mean $\pm$ SD (n = 3).

*II.C.5.b. Memory Phenotype of ROR1-specific T cells*

Naïve ($T_N$) and central memory ($T_{CM}$) T cells have been associated with long-term CAR$^+$ T cell therapeutic efficacy due to their ability to achieve persistence *in vivo*.(131) Both ROR1RCD28 and ROR1RCD137 cells predominantly expressed memory markers associated with $T_N$ and $T_{CM}$ memory phenotypes at Day 29 of co-culture (**Figure 13**). The mRNA expression of memory-associated genes was first evaluated with LCA, which identified a significant reduction in the inhibitory regulatory gene CTLA4 and an increase in expression of the transcription factor Lef1, which has been described to participate in CD8$^+$ T cell memory formation, in ROR1RCD137 cells relative to ROR1RCD28 cells (**Figure 13a**).(119, 288) As seen with the mRNA gene expression data, surface protein expression of CD28 was significantly (p = 0.003; Student's paired, 2-tailed t-test) higher in ROR1RCD137 cells compared to ROR1RCD28, whereas CD27 was highly expressed in both CAR$^+$ T cell populations suggesting they have not reached terminal differentiation (**Figure 13a, 13b, and 13d**). CAR$^+$ T cells populations were also similar in their high surface protein expression of lymphoid organ homing and memory markers CD62L and CCR7, suggesting they could home to organs harboring leukemia (**Figure 13a, 13c, and 13d**). A trend of decreased gene expression of SELL (CD62L) gene was observed in ROR1RCD137 cells, whereas CCR7 transcripts were roughly equivalent between the two CAR populations and protein expression was roughly equivalent for both sets as well. There was also a trend of higher expression of the antigen-experienced marker CD45RO over the more naïve-associated marker CD45RA in both populations (**Figure 13d**). Both groups were similar overall (p = 0.251; Two-way ANOVA) in expression of CD27, CD28, CD45RA,

CD45RO, CD62L, and CCR7. To further analyze memory potential, multi-parameter gating was used to define specific memory populations as naïve ($T_N$; $CD45RA^+CD27^+CD28^+CCR7^+$), central memory ($T_{CM}$; $CD45RA^{neg}CD27^+CD28^+CCR7^+$), effector memory ($T_{EM}$; $CD45RA^{neg}CD27^+CD28^{neg}CCR7^{neg}$), and effector memory RA ($T_{EMRA}$; $CD45RA^+CD27^{neg}CD28^{neg}CCR7^{neg}$).(131, 289) Most CAR$^+$ T cells belonged to $T_N$ and $T_{CM}$ groups with few $T_{EM}$ and $T_{EMRA}$ (**Figure 13e**). ROR1RCD137 had a trend of higher frequencies of cells belonging to $T_N$ and significantly higher $T_{CM}$ groups than ROR1RCD28, and overall the two CAR$^+$ T cell populations were different (p = 0.019; Two-way ANOVA). In aggregate, the surface phenotypes of ROR1-specific CAR T cells suggest their potential for memory and effector functions against ROR1$^+$ malignancies.
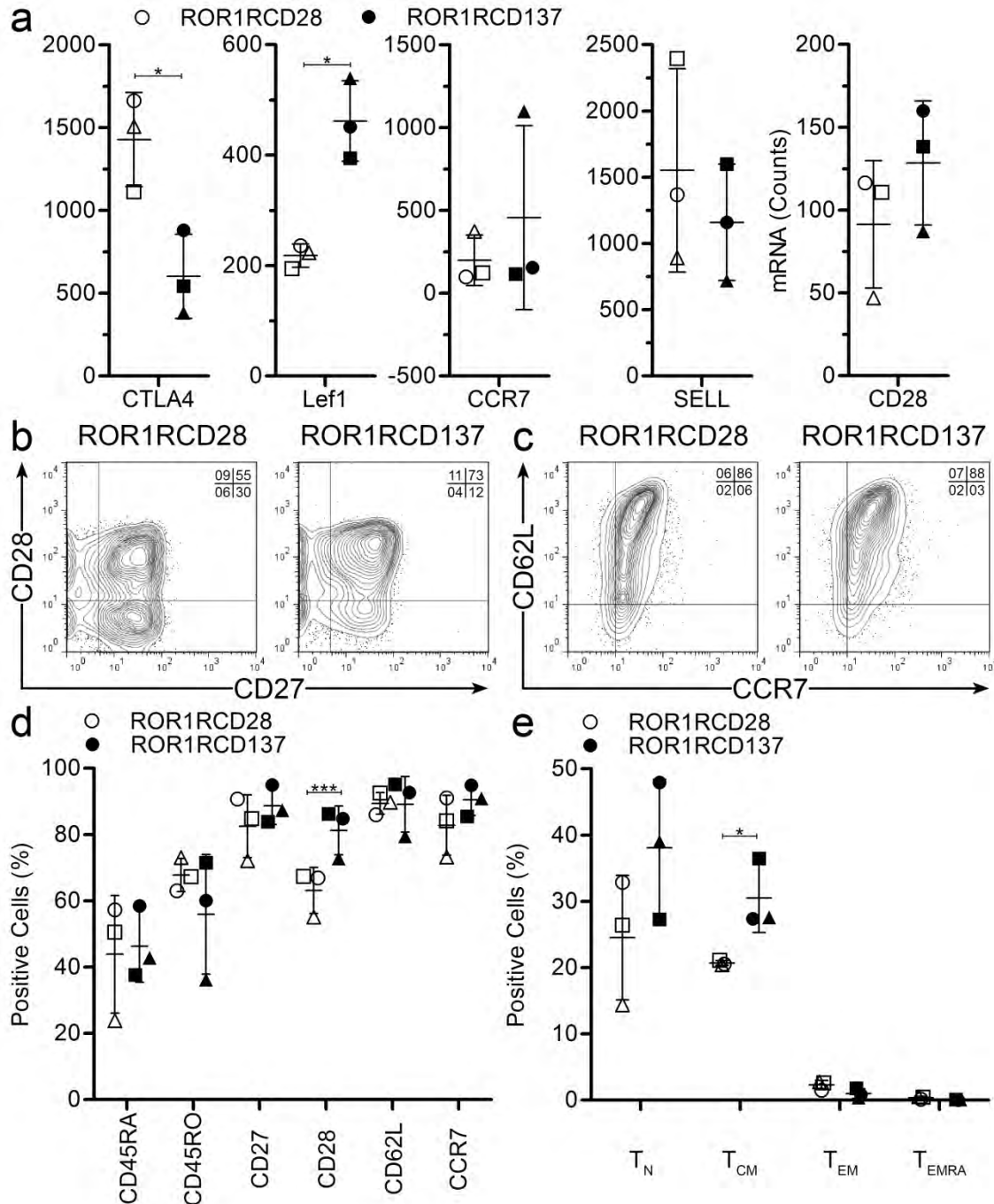
**Figure 13. Memory Markers on CAR+ T cell Surfaces.** After 29 days of expansion on clone#1 aAPC, ROR1RCD28 and ROR1RCD137 cells were (i) lysed for mRNA expression analysis or (ii) phenotyped for T cell surface markers by flow cytometry. (**a**) RNA lysates were run on the nCounter LCA and normalized expression of CTLA4 (far left), Lef1 (middle left), CCR7 (center), SELL (CD62L; middle right), and CD28 (far right) are displayed. Student's paired, 2-tailed t-tests were done for statistical analyses. *p<0.05 (**b**) CD27 (x-axes) and CD28 (y-axes) expression and (**c**) CCR7 (x-axes) and CD62L (y-axes) expression of one of 3 representative donors. (**d**) Frequencies of cells

staining positive for each memory marker. Student's paired, 2-tailed t-tests were done for statistical analyses. ***$p<0.001$ (**e**) Frequencies of cells staining positive for memory groups ($T_N$: naïve, $T_{CM}$: central memory, $T_{EM}$: effector memory, $T_{EMRA}$: effector memory RA). Statistical analysis was Student's paired, 1-tailed t-test between CAR groups for each memory group. *$p<0.05$ For (**a**), (**d**) and (**e**), each shape represents an individual donor, ROR1RCD28 are in open shapes and ROR1RCD137 are in closed shapes, and data are mean $\pm$ SD (n = 3).

II.C.6. TCR Repertoire of ROR1-specific T cells

Multiplex gene expression analysis was used to assay differences in TCR genes. Skewing towards a particular TCR clonotype was evaluated between the two CAR populations to assess whether CD28 or CD137 CARs particularly expand a select group of TCRs (**Figure 14**). The "direct TCR expression array" or DTEA was developed to analyze all 45 V$\alpha$ and 46 V$\beta$ TCR isotypes in a single reaction using the nCounter gene multiplex array platform.(290) After 22 days of expansion on clone#1 aAPC, ROR1RCD28 and ROR1RCD137 were assessed for TCR isotype expression by DTEA (**Figure 14**). Frequencies of TCR$\alpha$ regions were not statistically different between the two CARs (p = 0.25; Repeated measures Two-way ANOVA), no obvious trends were observed, and comparisons for each TCR$\alpha$ (Student's paired, two-tailed t-test) resulted in p values >0.05 for all alleles (**Figure 14a**). Similarly, TCR$\beta$ isotypes were not significantly different between ROR1RCD28 and ROR1RCD137 when analyzed together (p = 0.33) or as individual genes (**Figure 14b**). TCR$\alpha$ and TCR$\beta$ were both polyclonal suggesting that skewing to a particular TCR isotype did not occur. Additionally, DTEA measured TCR$\gamma$ and TCR$\delta$ expression where all V$\delta$ counts were 0.9% and 1.9% of the ROR1RCD28 and ROR1RCD137 total TCR frequencies, respectively, and V$\gamma$ counts were 6.2% and 8.1% of the ROR1RCD28 and ROR1RCD137 total TCR frequencies, respectively. These results showed that $\gamma\delta$ T cells were minor contributors to the total CAR$^+$ T cell pools, which were mainly $\alpha\beta$ T cells as determined by DTEA. Thus, CAR endodomain signaling was not preferential to a particular TCR$\alpha\beta$ clonotype but rather generated polyclonal $\alpha\beta$ T cells.
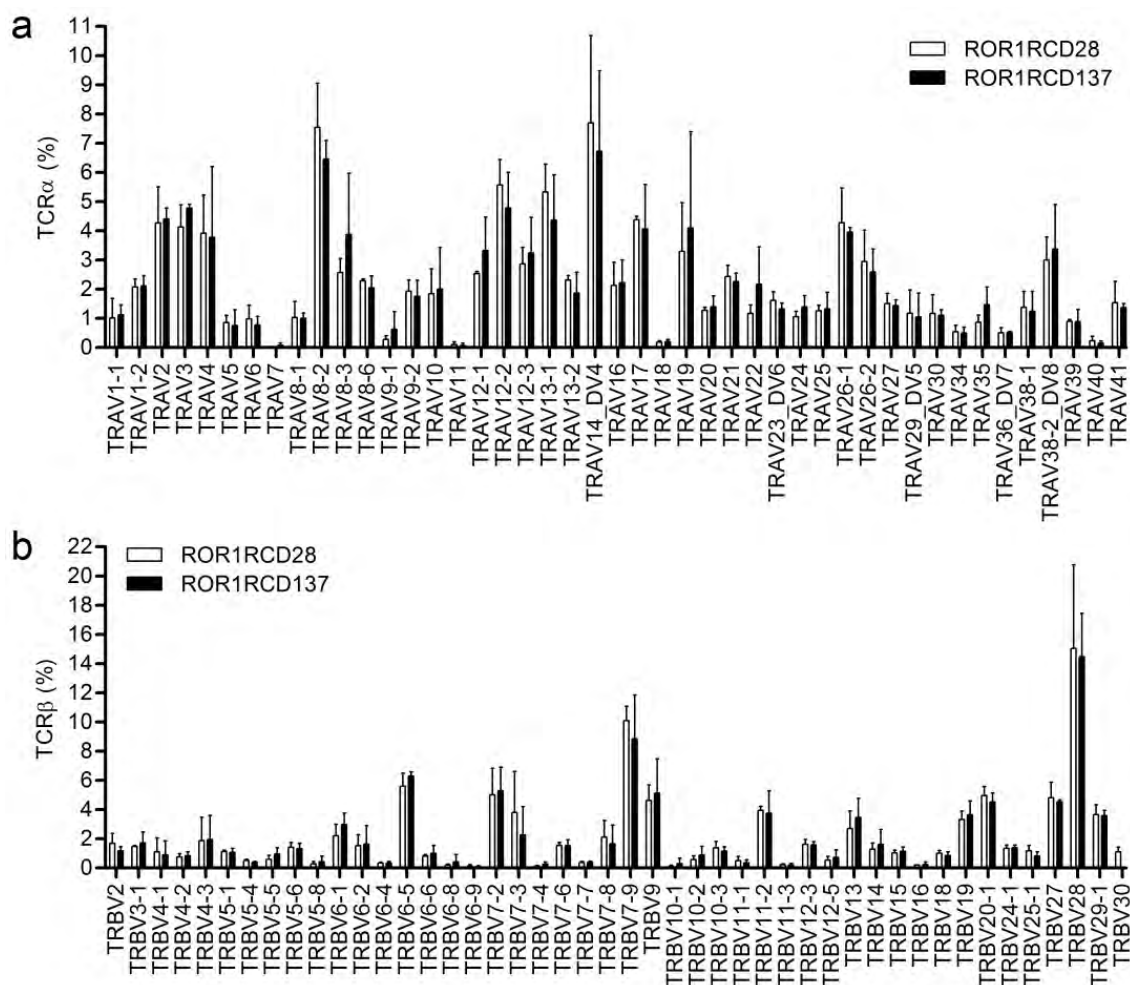
**Figure 14. TCRα and TCRβ Expression in ROR1RCD28 and ROR1RCD137 cells.** nCounter gene multiplex array was used to interrogate TCR isotype expression with "direct TCR expression array" (DTEA) in CAR-modified T cells after expansion on clone#1 aAPC. Cells were lysed at day 22 of co-culture period. (**a**) TCRα and (**b**) TCRβ expression in ROR1RCD28 (filled bars) and ROR1RCD137 (open bars) T cells.

## II.C.7. IFNγ Production by CAR^{+} T cells in Response to ROR1

In order to assess whether CAR^{+} T cells were functional and specific for ROR1^{+} tumor cells, IFNγ production was measured by flow cytometry after activation with leukemia cells or TCR agonists. Brefeldin-A was co-cultured with T cells to inhibit IFNγ secretion. Collectively, the data suggest that CAR^{+} T cells were specific and functional in responding to ROR1^{+} tumors.

### II.C.7.a. TCR Stimulus with Leukocyte Activation Cocktail

Phorbol myristate acetate (PMA) and Ionomycin were used as leukocyte activation cocktail (LAC) to stimulate the T cells for evaluation of maximal TCR response. LAC mimics TCR activation by activating protein kinase C (PKC) and increasing intracellular $Ca^{2+}$ levels and, therefore, is a measure of non-specific T cell activation.(291, 292) ROR1RCD28 and ROR1RCD137 T cells were mock activated (media only) as a negative control or activated with LAC for 6 hours. Significant expression of IFNγ was measured in response to LAC as seen in example histograms (**Figure 15a**) and average mean fluorescence intensities (MFI) of IFNγ staining (**Figure 15b**). There was a trend of higher production of IFNγ by ROR1RCD28 compared to ROR1RCD137 that was not statistically different ($p = 0.120$). These results established that IFNγ was produced when CAR^{+} T cells were activated through canonical TCR signaling pathways and suggested that ROR1RCD28 had higher propensity to express IFNγ relative to ROR1RCD137 cells.

*II.C.7.b. Specific IFNγ Production to ROR1$^+$ Leukemia Cells*

Both Kasumi2 and NALM6 are B-cell ALL cell lines that express CD19, but only Kasumi2 expresses ROR1 (**Figure 6a**). Thus, they were used to assess responsiveness of CAR$^+$ T cells to human leukemia cells in 6 hours of co-culture. As expected, ROR1RCD28 and ROR1RCD137 T cells produced IFNγ when co-cultured with Kasumi2 cells but not with NALM6 (**Figure 15c**). Similarly to LAC activation, ROR1RCD137 cells produced less IFNγ than ROR1RCD28 cells (**Figure 15d**) in response to the ROR1$^+$ cell line. Nonetheless, ROR1-specific CAR$^+$ T cells responded specifically to ROR1$^+$ leukemia.

*II.C.7.c. CAR$^+$ T cells Produce IFNγ in Response to Primary ROR1$^+$ Leukemia Cells but not Healthy ROR1$^{neg}$ B cell LCL*

It was important to ensure that ROR1-specific T cells would respond to primary ROR1$^+$ leukemia samples and spare normal B cells. LCL cell lines are immortalized healthy B cells, which served as negative controls in experiments where primary patient samples were used as targets. No IFNγ was produced by CAR$^+$ T cells when co-cultured for 6 hours with allogeneic LCL cell lines (**Figure 15e**). In contrast, significant (p = 0.004, Student's paired, 2-tailed t-test) IFNγ was produced by ROR1RCD28 and there was a trend of increased IFNγ production by ROR1RCD137 with CLL but did not reach a measure for statistical significance (**Figure 15e and 15f**). This was the same observation seen in an independent study testing ROR1-specific T cells, albeit with CARs derived from different mAbs specific for ROR1, where less cytokine production

was seen with CARs signaling through CD137 relative to those signaling through CD28.(199) Thus, ROR1-specific CAR^{+} T cells were functionally responsive to primary ROR1^{+} leukemia and not to healthy B cells.
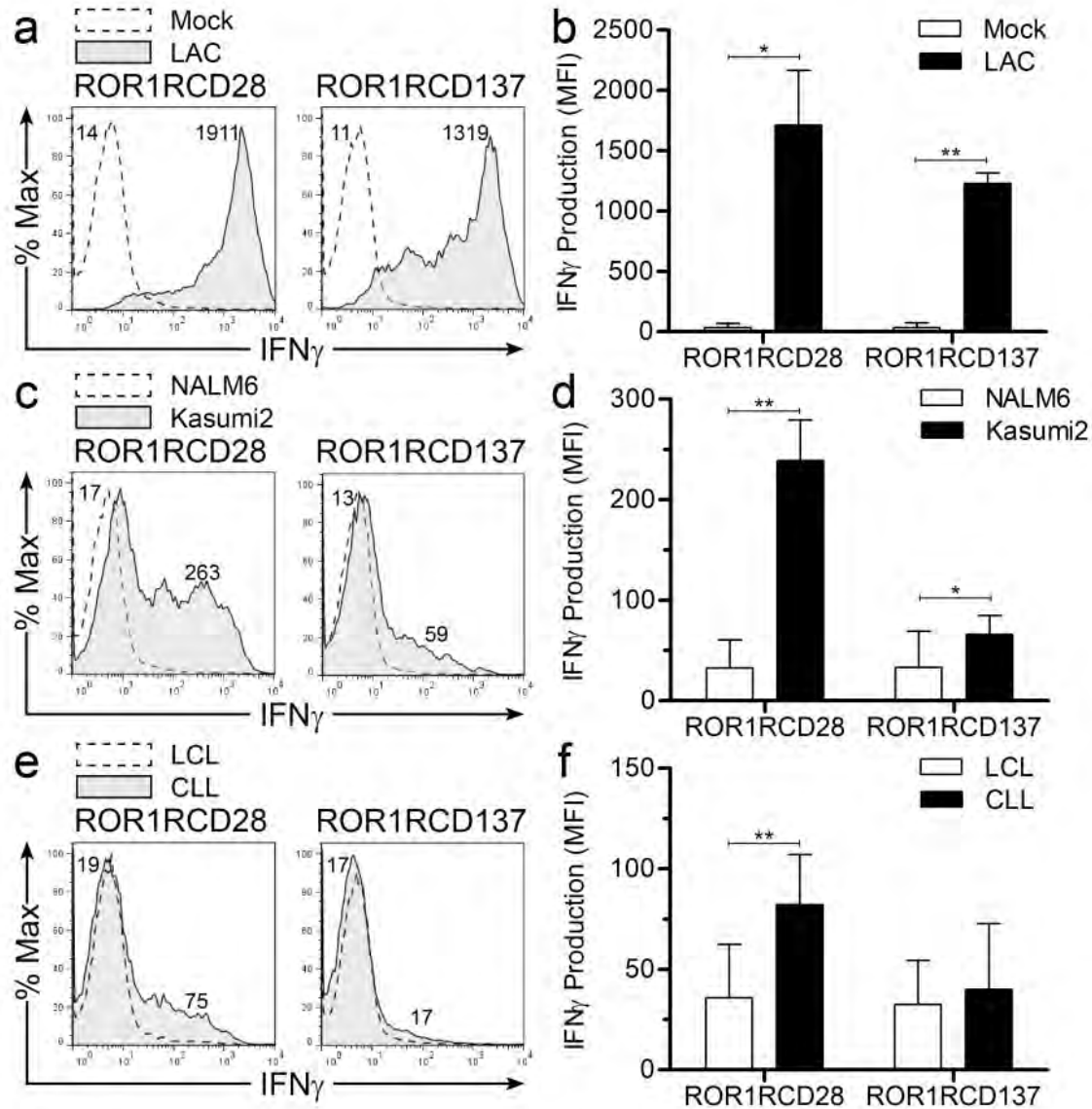
**Figure 15. IFNγ Production by ROR1-specific T cells in Response to ROR1$^+$ Targets.** Brefeldin-A (GolgiPlug) was added to T cells to block IFNγ secretion in order to measure functional responses to agonistic stimulation. At day 29 of co-culture, CAR$^+$ T cells were co-cultured for 6 hours at 37$^o$C and cells were gated for CD3$^+$Fc$^+$ to assess CAR responses to: (**a**)/(**b**) complete media (Mock) or PMA and Ionomycin (leukocyte activation cocktail; LAC), (**c**)/(**d**) B-ALL cell lines NALM6 (ROR1$^{neg}$) or Kasumi2 (ROR1$^+$), or (**e**)/(**f**) healthy donor LCL cell line (ROR1$^{neg}$) or CLL patient sample (ROR1$^+$). Mean fluorescence intensities (MFI) are displayed next to histograms in (**a**), (**c**), and (**e**), which are representative of three CAR$^+$ T cell donors. Mean ± SD (n = 3) are displayed in (**b**), (**d**), and (**f**). Student's paired, 1-tailed t-test for statistical analysis. *p<0.05 and **p<0.01

II.C.8. ROR1-specific Cytotoxicity by CAR$^+$ T cells

Cytotoxicity was another important assessment of ROR1-specific CAR$^+$ T cell function. Four-hour chromium release assays (CRA) are the gold-standard technique for *in vitro* killing assays. Thus, CRA was used to test specific lysis of ROR1$^+$ control cells, established tumor cell lines, and primary tumor cells. Significant lysis was only observed against ROR1$^+$ cells suggested that CAR$^+$ T cells were specific in their lytic abilities.

*II.C.8.a. CAR$^+$ T cells Lyse Leukemia but not Healthy B cells*

The clinical trial based on these data will treat patients with B-cell CLL, so primary B-cell CLL samples were tested as targets by allogeneic ROR1-specific CAR$^+$ T cells. ROR1$^{neg}$ LCLs were used for negative controls for CLL samples (**Figure 6a**). As expected, minimal lysis was observed by ROR1RCD28 and ROR1RCD137 against LCL (**Figure 16a**). In contrast, both ROR1RCD28 and ROR1RCD137 killed patient CLL cells in a dose-dependent manner (**Figure 16b**). More variability was observed in ROR1RCD28 samples in their lysis of CLL compared to ROR1RCD137, which was almost identical amongst donors. These data indicated specific lysis of ROR1$^+$ leukemia by CAR$^+$ T cells while sparing normal B cells.
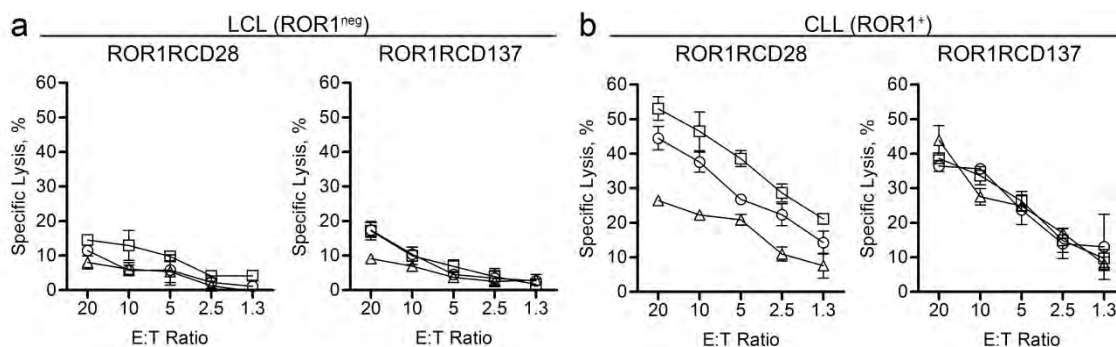
**Figure 16. Specific Cytolysis of Primary ROR1^{+} B-cell CLL by CAR^{+} T cells. (a)** Established ROR1^{neg} B-cell LCL and (**b**) Primary patient ROR1^{+} CLL cells were tested for cytolysis by ROR1-specific CAR^{+} T cells in standard 4-hour CRA. Specific lysis by ROR1RCD28 (left) and ROR1RCD137 (right) at decreasing effector to target (E:T) ratios. Each line and shape represents a different effector donor. Data are mean ± SD of triplicate measurements in CRA.

*II.C.8.b. ROR1-restricted Killing of Tumor Cell Lines*

A number of established tumor cell lines express ROR1 as an endogenous or introduced protein (**Figure 6**), so they were used for killing assays in parallel to cell lines lacking ROR1 expression. As expected, both ROR1RCD28 and ROR1RCD137 efficiently lysed EL4-ROR1$^+$ but showed minimal lysis of EL4-ROR1$^{neg}$ cells (**Figure 17a**). Similar to EL4 data, ROR1$^+$ B-ALL cell line Kasumi2 was lysed at significantly higher levels ($p < 0.0001$) compared to ROR1$^{neg}$ B-ALL cell line NALM6 by ROR1RCD28 (**Figure 17b left**). The same was observed for ROR1RCD137 where Kasumi2 was lysed at significantly higher levels ($p < 0.0001$) compared to NALM6 (**Figure 17b right**). In contrast to ROR1-specific CAR$^+$ T cells, donor-matched CD19$^+$ specific CAR$^+$ T cells lysed all three cell lines, which were all CD19$^+$ (data not shown), and suggested that ROR1RCD28 and ROR1RCD137 were more discriminant in their killing abilities. Furthermore, ROR1$^+$ OvCa cell line EFO27 was lysed at significantly ($p<0.0001$) higher levels than ROR1$^{neg}$ OvCa cell line A2780 by both ROR1RCD28 and ROR1RCD137 (**Figure 17c**). In summary, ROR1-specific CAR$^+$ T cells demonstrated effective and specific lysis of ROR1$^+$ tumor cells *in vitro*.
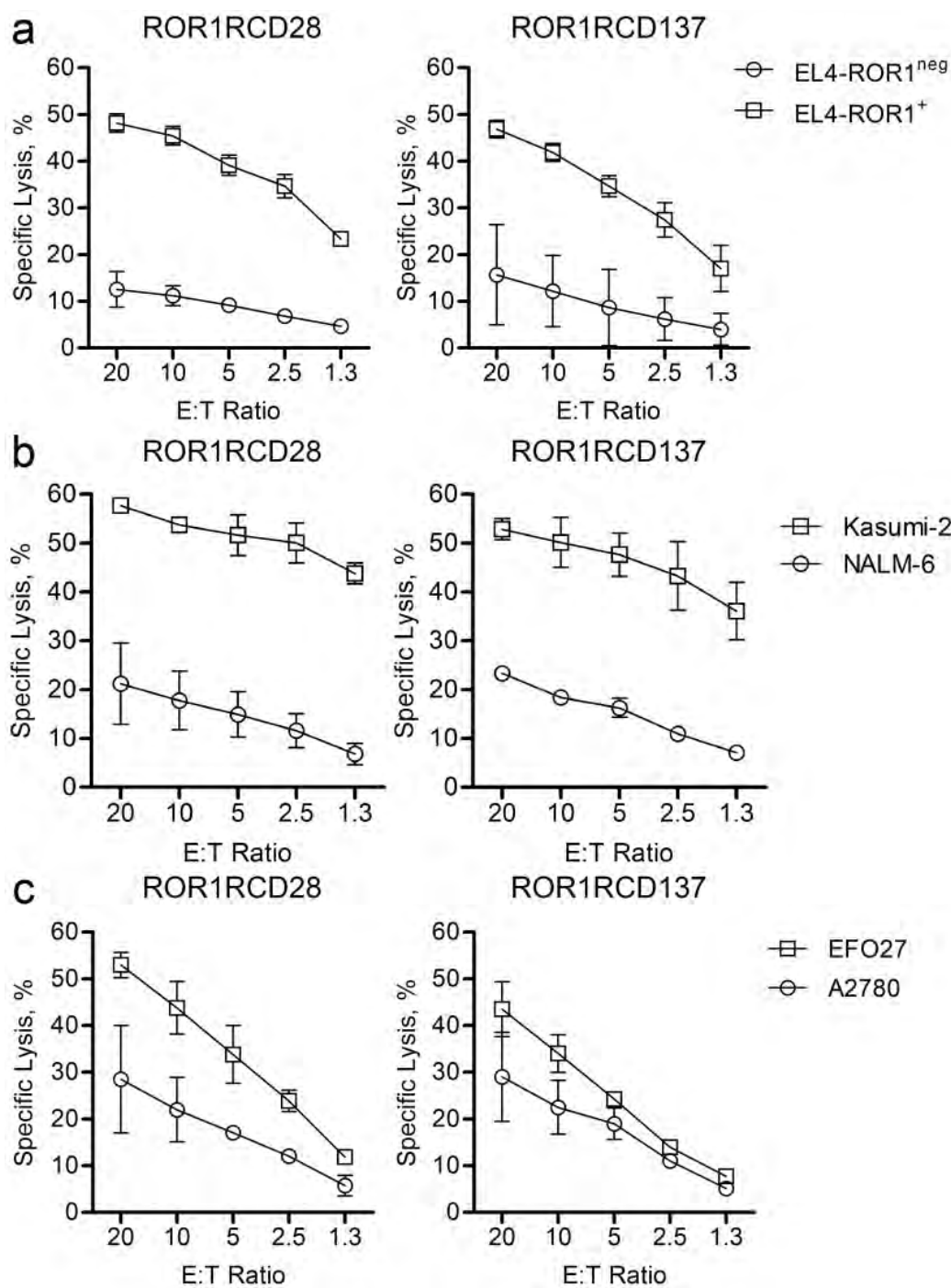
**Figure 17. Specific Cytolysis of Established ROR1$^+$ Tumor Cell Lines by CAR$^+$ T cells.** Standard 4-hour CRA were used to assess specific lysis of (**a**) EL4-ROR1$^{neg}$ (circles) or EL4-ROR1$^+$ (squares) cells, (**b**) ROR1$^{neg}$ NALM6 (circles) or ROR1$^+$ Kasumi2 (squares) cells, and (**c**) ROR1$^{neg}$ A2780 (circles) or ROR1$^+$ EFO27 (squares) cells by ROR1RCD28 (left) and ROR1RCD137 (right) at decreasing E:T ratios. Each line and shape represents a different target where data are mean ± SD of three donors with triplicate measurements in CRA.

80

II.C.9. *In Vivo* Leukemia Clearance by ROR1-specific T cells

In order to test the anti-tumor activity of ROR1-specific CAR$^+$ T cells *in vivo*, a mouse model of MRD was implemented for leukemia and ROR1-specific CAR$^+$ T cells were tested as treatment arms. Kasumi2 cells were sensitive to ROR1-specific T cells lysis, so they were genetically modified to express mKate red fluorescence protein to sort transduced cells (**Figure 18a**) and *Firefly Luciferase* (*ffLuc*; bioluminescence reporter) for non-invasive bioluminescence imaging (BLI) of tumor burden *in vivo* (**Figure 18b**). NOD.*scid*.$\gamma_c^{-/-}$ (NSG) mice were used because they lack functional adaptive immune systems and can, therefore, accept human tumor xenografts well. Mice engrafted with Kasumi2-*ffLuc*-mKate had consistent $\log_{10}$-fold increases in bioluminescence flux from their tumors and succumbed to disease after 27 (average) days after engraftment (**Figure 18c circles and 18d top panel**). ROR1RCD28 was able to diminish tumor burden significantly (p = 0.0004) above untreated mice as measured by tumor BLI flux (**Figure 18c squares and 18d middle panel**) and was able to increase survival significantly (p = 0.002) to an average of 30 days post-engraftment. Furthermore, ROR1RCD137 eliminated tumor burden significantly above both untreated mice (p = 0.0001) and ROR1RCD28-treated mice (p = 0.002) as measured by tumor BLI flux (**Figure 18c triangles and 18d bottom panels**), and was able to increase survival significantly longer compared to both untreated mice (p < 0.001) and ROR1RCD28-treated mice (p = 0.03) to 34 days (average) post-engraftment and up to 11 days relative to the first mouse that died in the untreated group and the last mouse that died in the ROR1RCD137 group. ROR1RCD137 cells had consistently lower frequencies of CAR$^+$ T cells (94%, 62%, and 46% at doses 1, 2, and 3, respectively) prior to infusion relative

to ROR1RCD28 cells, which expressed CAR at >90% for all three doses. The T cell doses were given based as $10^7$ total cells/mouse, so a greater anti-tumor effect was seen with ROR1RCD137 with fewer total CAR$^+$ T cells, which highlights their ability to outperform ROR1RCD28 in tumor killing *in vivo*. In summary, ROR1-specific CAR$^+$ T cells can efficiently treat ROR1$^+$ leukemia and, therefore, can now be moved into the clinic for testing in patients with ROR1$^+$ malignancies.
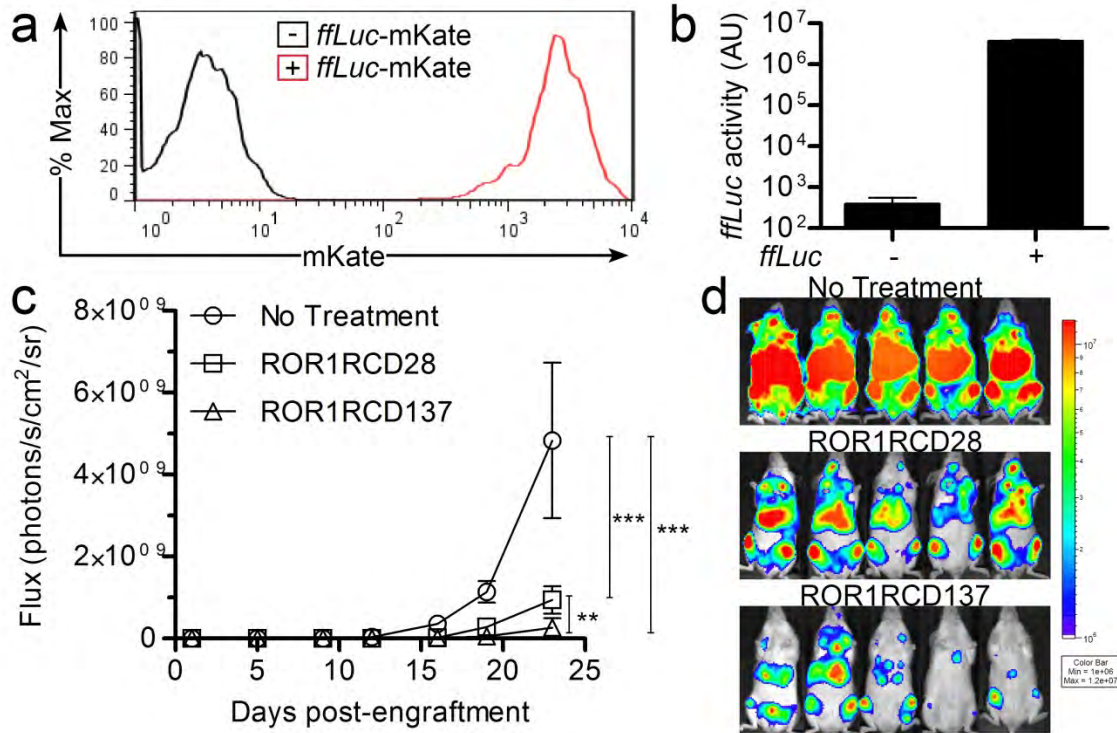
**Figure 18. *In vivo* Tumor Clearance by ROR1-specific CAR⁺ T cells.** ROR1⁺ B-ALL cell line Kasumi2 was transduced with mKate-*ffLuc* lentiviral particles and cells were sorted for uniform mKate expression by FACS. (**a**) mKate expression in parental cell line (black histogram) or transduced cell line (red histogram). (**b**) *In vitro* luciferase activity of parental Kasumi2 cell line (without *ffLuc*) and transduced Kasumi2-*ffLuc*-mKate cells. NSG mice were engrafted with $4 \times 10^4$ Kasumi2-*ffLuc*-mKate cells intravenously (i.v.) and were treated with three doses of $10^7$ T cells i.v. to assess the ability of ROR1-specific T cells to manage MRD. High dose (60 kIU) IL2 was given intraperitoneally (i.p.) the day of T cell dosing and the following day. (**c**) Non-invasive bioluminescence imaging (BLI) flux kinetics during experiment where untreated mice are in circles, ROR1RCD28-treated mice are in squares, and ROR1RCD137-treated mice are in triangles. Two-way ANOVA was used for statistical analysis. \*\*$p<0.01$ and \*\*\*$p<0.001$ (**d**) Representative BLI images at day +23 post-engraftment.

## II.D. Discussion

### II.D.1. Importance of Developing ROR1-specific T cells for Leukemia Patients

This work aimed to develop pre-clinical data to support a "first-in-man" Phase I clinical trial of ROR1-specific T cell treatments for ROR1^{+} malignancies. The major advantage of this therapy over the current anti-CD19 cellular therapies is that normal B cells would be spared when targeting ROR1 as CD19 is uniformly expressed on most B cells and is required for B cell function.(56, 57) B cells are the primary arm of the humoral response and are critical for the adaptive immune response in clearance of microbial pathogens.(89) However, people can survive without B cells, albeit under threat of novel pathogens, if they receive serum immunoglobulin replacement therapy.(63) Thus, quality of life would be certainly improved if CAR[+] T cell therapy patients had a normal repertoire of healthy B cells as would be achieved by targeting ROR1 instead of CD19.

### II.D.2. ROR1 as a Tumor Target and Safety Concerns in Immunotherapy

ROR1 was originally identified on the surface of CLL cells with absent expression on normal tissues, including cells in the hematopoietic compartment.(66, 75) Subsequently, ROR1 has been described on t(1;19) B-ALL and a number of solid tumors, e.g. breast, ovarian, and pancreatic cancers.(13, 67, 79) Some expression of ROR1 mRNA species was identified in normal lung, pancreas, and adipose tissue, by qPCR of healthy donor tissue panels, and protein expression was later corroborated on the cell surface in adipocytes and in the cytoplasm in pancreatic islet cells and alveolar macrophages by immunohistochemical staining with the 2A2 ROR1-specific antibody.(77, 81) However,

this antibody also displayed cytosolic staining of a number of tissues that do not express ROR1 mRNA transcripts, e.g. adrenal glands, cardiac muscle, neurons, colon, endometrium, hypophysis, larynx, liver, ovary, salivary, small intestine, skin, stomach, and thymus, which means that (i) the mRNA expression data is inaccurate or (ii) the 2A2 antibody is not completely specific for ROR1. Testing of the R12 goat antibody specific for ROR1 binding to normal tissues has not yet been reported.(293) In contrast to the 2A2 data, RNAseq analysis did not corroborate ROR1 mRNA presence in normal healthy tissues (Kipps TJ, UCSD, unpublished observations). Moreover, the 4A5 ROR1-specific mAb from which the CAR was developed in this study did not detect ROR1 in healthy tissues by both Western blot and immunohistochemistry.(67, 75) The only reported staining of ROR1 with the 4A5 mAb outside of malignancies was described on hematogones, which are B-cell precursors, and loss of hematogones would impact B cell differentiation but not the mature B cell pool.(66) There is always the risk of potential "on-target/off-target" toxicity of proper antigen recognition by CAR$^+$ T cells on undesired tissues expressing low levels of antigen, but we are confident that our approach is safe because (i) 4A5 did not stain normal tissue and the CAR was derived from this Ab, (ii) homing to pancreas and or adipose tissue is unlikely given the homing repertoire expressed by CAR$^+$ T cells which predicted for homing to lymphoid organs (CCR7 and CD62L), and (iii) high tumor burden in many CLL patients will likely be seen first and occupy the T cells from other organs. As a control for adverse events, suicide genes, e.g. inducible Caspase9, can be co-expressed with CAR in order to eliminate T cells *in vivo* with drugs specific for the suicide gene of choice.(294) In the

end, these questions will only be answered once clinical trials test these hypotheses in humans.

## II.D.3. CD28 versus CD137 in CAR Design

A common debate in CAR immunotherapy at present is whether to use CD28 endodomain, as most investigators have done, or CD137 endodomain, both of which have led to objective clinical responses.(4-7, 32) A direct comparison of CD28 versus CD137 signaling in CD19-specific CARs developed at MDACC (and analogous to the ROR1-specific CARs in design) resulted in almost indistinguishable characteristics *in vitro* but CD137 was superior *in vivo* in leukemia clearance compared to CD28 (Singh H, unpublished observations). In this study, the most notable differences between the two ROR1-specific CARs were in (i) memory phenotype, (ii) *in vitro* IFNγ production, and (iii) *in vivo* tumor clearance. In regards to surface phenotype, both ROR1RCD28 and ROR1RCD137 T cells were almost completely naïve ($T_N$) and central memory T cells ($T_{CM}$) after *ex vivo* expansion, and there were more of both $T_N$ and $T_{CM}$ populations in ROR1RCD137 cells (**Figure 13**). Indeed, both of these populations have been correlated to limited effector functions including reduced cytokine production and cytolysis.(132, 135) It is consistent then that ROR1RCD137 cells produced less IFNγ when challenged with ROR1^{+} targets (**Figures 15**), and fewer cytokine mRNA transcripts were produced by ROR1RCD137 relative to ROR1RCD28 as evaluated by nCounter LCA (data not shown). Indeed, the ability to produce cytokines was inversely correlated with CD8^{+} T cell efficacy in other T cell immunotherapies.(295) Again,

reduced cytokine production was also observed with ROR1-specific CARs signaling through CD137 that were derived from the 2A2 mAb and its higher affinity counterpart R12 mAb.(199) Similar killing was detected by both CAR populations against ROR1$^+$ targets, with a minor exception of primary cell lines where ROR1RCD28 was highly variable in cytolysis between donors and exceeded ROR1RCD137 in killing for 2 out of 3 donors (**Figure 16b**). In contrast to the *in vitro* results, ROR1RCD137 was significantly (p = 0.0001) better at eliminating ROR1$^+$ leukemia compared to ROR1RCD28, which was significantly better (p < 0.0001) than no treatment (**Figure 18**). Furthermore, these results were achieved with fewer total CAR$^+$ T cells infused into each mouse, because the same total number was injected but CAR percentage was lower in ROR1RCD137 relative to ROR1RCD28. Possible explanations of the differences are (i) higher frequencies of $T_N$ and $T_{CM}$ memory cells that are correlated with highest CAR$^+$ T cell responses relative to other classification,(131) (ii) lower expression of inhibitory molecules like CTLA4 (**Figure 13**), (iii) production of other inflammatory molecules other than IFNγ such as IL17, and/or (iv) longer persistence in the mice which has been correlated to memory formation and increased anti-tumor activity.(6, 189, 215, 237) The NSG mice used for *in vivo* studies lack human homeostatic cytokines, e.g. IL7 and IL15, that can improve persistence in patients treated with ROR1-specific T cells and therefore increase the potential of the anti-tumor effects observed in the mouse studies. A side-by-side comparison of the two CARs in clinical trials will be the ultimate test of which CAR is better for cancer treatment.

II.D.4. Immediate Plans for ROR1-specific T cells in Leukemia Treatment

A Phase I clinical trial has been approved by the NIH RAC and is in process for MD Anderson IRB approval. The trial design is to co-infuse ROR1RCD28 and ROR1RCD137 cells in a competitive repopulation experiment to maximize potential therapeutic efficacy and determine which CAR will persist longer in the patients. PCR will be used as a highly-sensitive means to detect persistence of one population over another based on unique oligonucleotides present in the two CAR transposons (SIM for CD28 and FRA for CD137). As this will be the first time ROR1-specific T cells are infused into humans, it is the primary endpoint to determine toxicity and maximum tolerated doses. There is strong evidence that this will work as means to eliminate leukemia while maintaining normal B cells, and will be the first time that ROR1 has been a target of immunotherapy for cancer treatment.

## CHAPTER III

**Bi-specific T cells Expressing Polyclonal Repertoire of Endogenous γδ T-cell Receptors and Introduced CD19-specific Chimeric Antigen Receptor**

### III.A. Hypothesis and Rationale

The *hypothesis* of this chapter is that enforced CAR expression on γδ T cells will stimulate them independent of their TCRγδ, thus leading to expansion of γδ T cells with polyclonal TCRγδ repertoire, and would amplify the anti-tumor effects from TCRγδ towards TAA$^+$ malignancies through the CAR. The *rationale* for this specific aim is that (i) γδ T cells have inherent anti-tumor immunity through a number of combinations of TCRγ and TCRδ pairings, (ii) the use of γδ T cells in the clinic is currently restricted to Vγ9Vδ2 even though other γδ T cell lineages have anti-tumor reactivity, (iii) CARs stimulate T cells independent of their TCR, (iv) electroporation of SB transposons containing the CAR can be achieved in quiescent PBMC with a polyclonal repertoire of γδ T cells, and (v) CD19-specific CAR transposon plasmids and CD19$^+$ aAPC are currently in clinical trials at MD Anderson and these reagents can be used to quickly translate findings from this chapter into clinical trials. Therefore, using a polyclonal set of γδ T cells for CAR-based immunotherapy would allow for targeting the tumor through both CAR and multiple TCRγδ pairings to maximize anti-tumor immunity through bi-specific T cells.

**III.B. Introduction**

"Chimeric antigen receptors (CARs) re-direct T-cell specificity to tumor-associated antigens (TAAs), such as CD19, independent of major histocompatibility complex (MHC).(57, 186, 189, 272, 296) This genetic modification of T cells has clinical applications as adoptive transfer of $CAR^+$ T cells with specificity for CD19 can lead to anti-tumor responses in patients with refractory B-cell malignancies.(6, 7, 32, 56) Current trials administer $CAR^+$ T cells co-expressing αβ T-cell receptor (TCRαβ) derived from a population that represents 95% of the peripheral T-cell pool. However, the remaining 1-5% of circulating T cells expressing TCRγδ (γδ T cells) have clinical appeal based on their endogenous cytotoxicity towards tumor cells as well as their ability to present TAA and elicit an anti-tumor response.(177, 297, 298) This population of T cells directly recognizes TAA, *e.g.*, heat shock proteins, MHC class I chain-related gene A/B (MICA/B), F1-ATPase, and intermediates in cholesterol metabolism (phosphoantigens), in humans.(299) Therefore, broad recognition of tumor cells and anti-tumor activity is achieved by these T cells expressing a diverse TCRγδ repertoire (combination of Vδ1,  Vδ2,  or Vδ3  with one of fourteen Vγ chains).(300)

More specifically, T cells expressing Vδ1 and Vδ2 have been associated with anti-tumor immunity, but current adoptive immunotherapy approaches are limited to the Vδ2 sub-population due to limited expansion methods of Vδ1 to clinically-sufficient numbers of cells for human applications. For the most part,

γδ T cells have been numerically expanded *in vivo* and *ex vivo* using Zoledronic acid (Zol),(301) an aminobisphosphonate that results in selective proliferation of T cells expressing Vγ9Vδ2 TCR.(175, 177, 297) This treatment modality has resulted in objective clinical responses against both solid and hematologic tumors, but has not been curative as a monotherapy. Vδ1 γδ T cells have not yet been infused, but their presence has correlated with complete responses observed in patients with B-cell acute lymphoblastic leukemia (B-ALL) after undergoing αβ T cell-depleted allogeneic hematopoietic stem-cell transplantation (HSCT).(302-305) As both of these sub-populations of γδ T cells are associated with anti-tumor activity, but have not been combined for cell therapy, we sought a clinically-appealing approach to propagate T cells that maintain a polyclonal TCRγδ repertoire.

Recognizing that a CD19-specific CAR can sustain the proliferation of αβ T cells on artificial antigen presenting cells (aAPC) independent of TCRαβ usage,(280) we hypothesized that CAR$^+$ γδ T cells would expand on aAPC independent of TCRγδ. Our approach was further stimulated by the observation that K562, the cell line from which the aAPC are derived, are a natural target for γδ T cells.(303) We report that CAR$^+$ γδ T cells can be propagated to clinically-relevant numbers on designer aAPC while maintaining a polyclonal population of TCRγδ as assessed by our "direct TCR expression assay" (DTEA), a novel digital multiplexed gene expression analysis that we adapted to interrogate all TCRγδ isotypes.(290) These CAR$^+$ γδ T cells

displayed enhanced killing of CD19$^+$ tumor cell lines *in vitro* compared to polyclonal γδ T cells not expressing CAR. Leukemia xenografts in immunocompromised mice were significantly reduced when treated with CAR$^+$ γδ T cells compared to control mice. This study highlights the ability of aAPC to numerically expand bi-specific T cells that exhibit introduced specificity for CD19 and retain endogenous polyclonal TCRγδ repertoire.

## III.C. Results

### III.C.1. CAR$^+$ γδ T cells Numerically Expand on aAPC

To date, it has been problematic to synchronously manipulate and expand multiple γδ T-cell subpopulations for application in humans. Viral-mediated gene transfer typically requires cell division to achieve stable gene transfer and CARs have been introduced into transduced T cells expressing just Vδ2 TCR following the use of aminobisphosphonates to drive proliferation.(306) In contrast, non-viral gene transfer with *Sleeping Beauty* (SB) transposition can be achieved in quiescent peripheral blood mononuclear cells (PBMC) with the full complement of peripheral γδ T cells initially present. Thus, stable expression of CAR can be achieved without prior T-cell propagation, enabling us to investigate if a population of T cells expressing polyclonal TCRγδ chains could then be numerically expanded in a CAR-dependent manner on designer artificial antigen presenting cells (aAPC). PBMC were electroporated (Day 0) with SB transposon/transposase system to enforce expression of a second generation

CD19-specific CAR (CD19RCD28)(57) that signals through chimeric CD28 and CD3ζ. Electroporated cells were sorted using paramagnetic beads to separate the 4.0% ± 1.5% (mean ± standard deviation (SD); n = 4) CAR$^+$ γδ T cells from the majority of CAR$^+$ αβ T cells. The CAR$^+$ γδ T cells were selectively propagated by the recursive additions of γ-irradiated K562-derived aAPC (clone #4, genetically modified to co-express CD19, CD64, CD86, CD137L, and membrane bound IL15)(57) with soluble IL2 and IL21. IL21 is included in the manufacture of our CAR$^+$ αβ T cells so it was used to propagate CAR$^+$ γδ T cells.(57) Prior experiments predicted that IL2 and IL15 enhance the proliferative potential of γδ T cells, and synergy between IL2 and IL21 has led to improved anti-tumor activity compared with γδ T cells grown with either IL2 or IL21 alone.(174, 178, 307-309) Sham electroporations were undertaken to provide staining control T cells that were propagated by cross-linking CD3 using aAPC loaded with OKT3 to numerically expand CAR$^{neg}$ αβ T cells.(310) As expected, CAR was expressed on the day following electroporation (Day 1) in most of the T cells, including γδ T cells, which comprised up to 10% of the mononuclear cells (**Figure 19a, left**). After 36 days of co-culture on aAPC, the majority of cells co-expressed CD3 and TCRγδ with 30.7% ± 23.3% (n = 4) CAR expression (**Figure 19a, right**). The absolute CAR proportions at Day 36 varied in frequency depending on the donor, but increased compared to the initial populations of CAR$^+$ γδ T cells at Day 1 (**Figure 19b**). As we have demonstrated, our aAPC co-culture system enforces CAR expression in αβ T cells (>90% CAR$^+$ T cells by 28 days of co-culture),(57) but the apparent lack of

the same degree of selective pressure when combined with γδ T cells was attributed to an inherent ability of CAR$^{neg}$ γδ T cells to sustain proliferation on aAPC derived from K562. Continuous proliferation of both CAR$^{neg}$ and CAR$^+$ γδ T cells was observed over the tissue culture period. Even so, we could generate up to $1.5x10^9 \pm 1.2x10^9$ (n = 3) CAR$^+$ γδ T cells from the $2.8x10^5 \pm 1.5x10^5$ (n = 3) CAR$^+$ γδ T cells at the start of the culture (**Figure 19c**). Most of the propagated cells co-expressed CD3 and TCRγδ, but did not express TCRαβ (**Figure 19d**). These data demonstrate that aAPC could be used to sustain proliferation of CAR$^+$ T cells co-expressing TCRγδ.
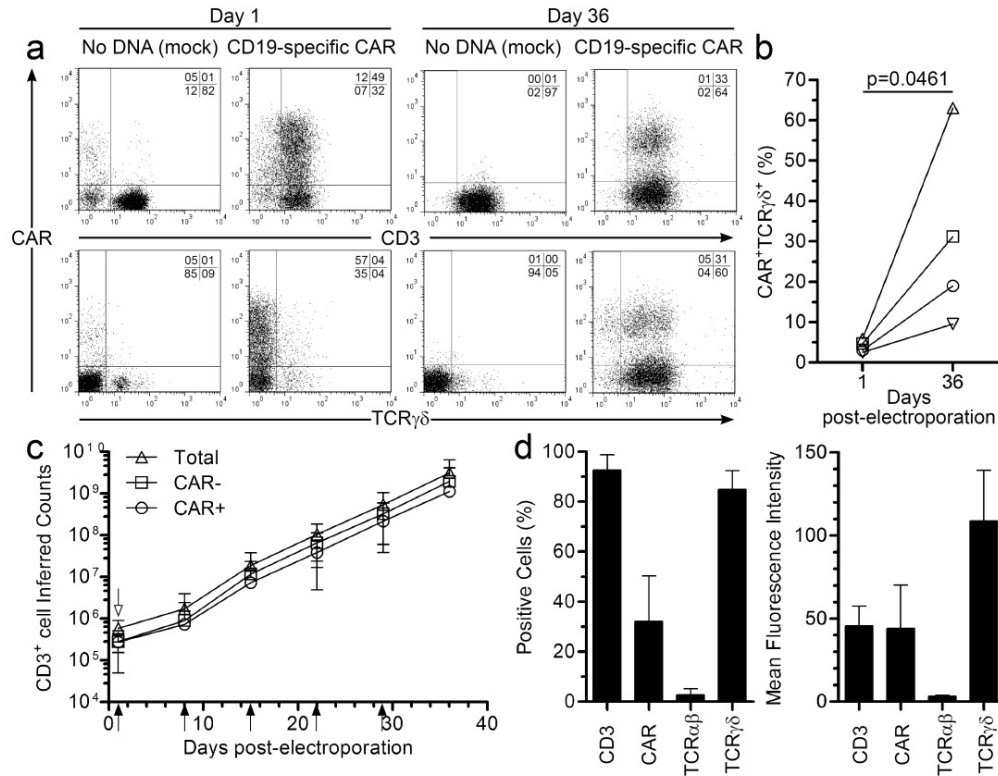
**Figure 19. CAR$^+$ γδ T cells Propagate on Designer aAPC. (a)** Transient (Day 1) and stable (Day 36) expression of CAR in T cells (top) and γδ T cells (bottom) in mock electroporated ("no DNA") or CD19-specific CAR electroporated cells (CD19RCD28). **(b)** Percentage of CAR$^+$ γδ T cells in the culture as transient (Day 1) and stable (Day 36) expression where each shape represents an individual donor. **(c)** Rate of expansion of total γδ T cells (triangles), CAR$^{neg}$ γδ T cells (squares), and CAR$^+$ γδ T cells (circles) over tissue culture period following paramagnetic bead sorting (open arrow) and recursive stimulation (closed arrows) with aAPC and exogenous IL2 and IL21 administration. **(d)** Percentage-positive cells and mean fluorescence intensity of CD3, CAR, TCRαβ, and TCRγδ at day 36. Data are mean ± SD (n = 4) and quadrant percentages of flow plots are in upper right corner. **This work was originally published in *Molecular Therapy*.** Deniger, D. C., K. Switzer, T. Mi, S. Maiti, L. Hurton, H. Singh, H. Huls, S. Olivares, D. A. Lee, R. E. Champlin, and L. J. Cooper. 2013. Bispecific T-cells Expressing Polyclonal Repertoire of Endogenous gammadelta T-cell Receptors and Introduced CD19-specific Chimeric Antigen Receptor. Mol Ther. 21(3): 638-647.(311) © **Nature Publishing Group**

III.C.2. Immunophenotype of Numerically Expanded CAR$^+$ γδ T cells

Multi-parameter flow cytometry was used to gate on CAR$^+$ T cells and analyze their expression of cell surface markers (**Figure 20**). TCRγδ was expressed at high and low densities (**Figure 20a, top**). CD56, a marker of MHC-unrestricted lytic ability,(287) was also expressed on T cells, but the culture contained <1% CD3$^{neg}$CD56$^+$ NK cells and <1% CD3$^+$Vα$_{25}$TCR$^+$ NKT cells (data not shown). In contrast to αβ T cells, no CAR$^+$ γδ T cells expressed CD4, some were CD8$^+$, but most were CD4$^{neg}$CD8$^{neg}$, which is consistent with what is known for γδ T cells.(286) The relative frequencies for each donor are shown in **Figure 20b**. Markers associated with memory, *e.g.*, CD27, CD28, CD62L, and CCR7, were expressed by CAR$^+$ γδ T cells (**Figure 20a, bottom**). Both naïve (CD45RA) and antigen-experienced (CD45RO) cells were present after propagation on aAPC, and the T cells were not exhausted as measured by low expression of CD57 (**Figure 20b**). In aggregate, cultures contained a heterogonous mixture of naïve (CD45RA$^+$CD27$^+$CD28$^+$CCR7$^+$; 26.5% ± 6.2%), central memory (CD45RA$^{neg}$CD27$^+$CD28$^+$CCR7$^+$; 7.8% ± 3.6%), effector memory (CD45RA$^{neg}$CD27$^+$CD28$^{neg}$CCR7$^{neg}$; 10.1% ± 5.4%), and EMRA (CD45RA$^+$CD27$^{neg}$CD28$^{neg}$CCR7$^{neg}$; 7.6% ± 3.4%) T-cell phenotypes.(131, 289) Co-stimulation by enforced expression of CD86 and CD137L (4-1BBL) on aAPC may be important for CAR$^+$ γδ T-cell numeric expansion due to expression of their receptors CD28 and CD137 (4-1BB), respectively. Molecules associated with homing to bone marrow (cutaneous lymphocyte antigen (CLA) and CXCR4) and lymph nodes (CD62L and CCR7) were present

on CAR$^+$ γδ T cells suggesting that they could migrate to sites known to harbor leukemia. In sum, propagated CAR$^+$ γδ T cells expressed T cell-associated surface markers that indicate desired potential for memory and homing.
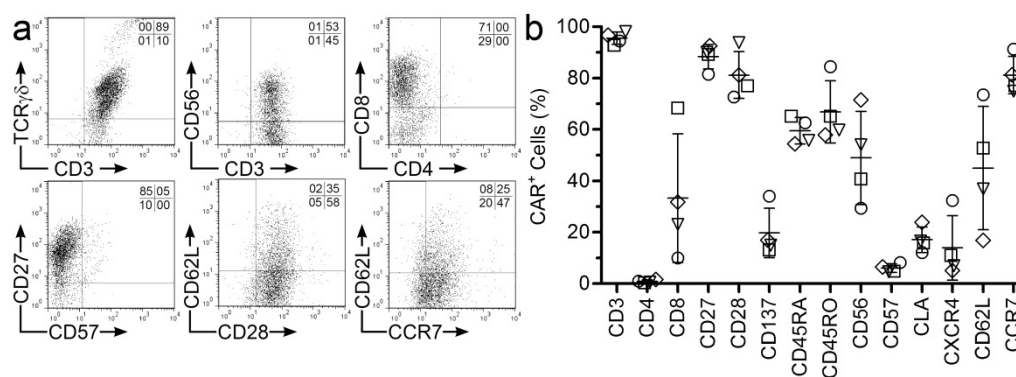
**Figure 20. Immunophenotype of Electroporated, Separated, and Propagated CAR⁺ γδ T cells.** (**a**) Expression by flow cytometry of cell-surface markers associated with T cells and memory as gated on CD3⁺CAR⁺ cells. (**b**) Percentages of CAR⁺ T cells expressing T-cell markers where each shape represents a different donor. Data are mean ± SD (n = 4). Quadrant percentages of flow plots are in upper right corner. **This work was originally published in** *Molecular Therapy*. Deniger, D. C., K. Switzer, T. Mi, S. Maiti, L. Hurton, H. Singh, H. Huls, S. Olivares, D. A. Lee, R. E. Champlin, and L. J. Cooper. 2013. Bispecific T-cells Expressing Polyclonal Repertoire of Endogenous gammadelta T-cell Receptors and Introduced CD19-specific Chimeric Antigen Receptor. Mol Ther. 21(3): 638-647.(311) © **Nature Publishing Group**

III.C.3. Direct TCR Expression Assay to Reveal $\gamma$ and $\delta$ TCR Usage in CAR$^+$ $\gamma\delta$ T cells

We sought to determine that aAPC-propagated CAR$^+$ T cells were indeed bi-specific as defined by the presence of a polyclonal population of TCR$\gamma\delta$ alleles. Up to now, it has been difficult to determine the pattern of expression of the $\gamma$ and $\delta$ TCR chains. Therefore, we adapted our DTEA to assess the complete TCR$\gamma\delta$ transcriptome. This approach takes advantage of the nCounter assay system to measure multiple bar-coded genes in a single reaction with high sensitivity and linearity across a broad range of expression.(312) A multiplexed CodeSet was designed with two sequence-specific probes for each allele to evaluate TCR$\gamma\delta$ isotypes. The DTEA was initially validated using Zol to preferentially propagate V$\gamma$9V$\delta$2 cells from PBMC and, as expected, the resultant TCR usage was dominated by both V$\delta$2 and V$\gamma$9 at protein (**Figure 21a**) and mRNA levels (**Figure 21b and 21c**). A second validation employed antibodies directed against $\gamma\delta$ T-cell subsets (V$\delta$1 and V$\delta$2; no commercially available antibodies to V$\delta$3) to measure their mRNA expression. V$\delta$1$^{neg}$V$\delta$2$^{neg}$, V$\delta$1$^+$V$\delta$2$^{neg}$, and V$\delta$1$^{neg}$V$\delta$2$^+$ cells were sorted from CAR$^{neg}$ T cells (to maximize the number of V$\delta$2 cells recovered by fluorescence-activated cell sorting, FACS) and subjected to DTEA (**Figure 22a**). As expected, V$\delta$1$^+$V$\delta$2$^{neg}$, V$\delta$1$^{neg}$V$\delta$2$^+$, and V$\delta$1$^{neg}$V$\delta$2$^{neg}$ expressed V$\delta$1*01, V$\delta$2*02, and V$\delta$3*01 mRNA species, respectively (**Figure 22b**). These two strategies supported the validity of the DTEA panel enabling the identity of TCR$\gamma\delta$ to be determined in CAR$^+$ T

cells. Therefore, we measured the mRNA levels for all three Vδ alleles as present in electroporated, separated, and propagated CAR$^+$ γδ T cells which correlated with multi-parameter flow cytometry on gated CAR$^+$ T cells to reveal the frequencies of Vδ subsets based on protein expression. The three Vδ populations were present in ascending frequency (Vδ1>Vδ3>>>Vδ2) in the electroporated and propagated T cells (**Figure 22c**). CAR$^{neg}$ γδ T cells displayed similar frequencies of Vδ TCR usage as CAR$^+$ γδ T cells. DTEA array also assessed Vγ usage, which is of particular utility because only one antibody against Vγ9 is commercially available, thus limiting the tools with which to detect Vγ usage. Of note, Vγ2, Vγ7, Vγ8 (both alleles), Vγ9, and Vγ10 were present in CAR$^+$ T-cell cultures (**Figure 22d**). A lack of commercially-available antibodies prevented assessment of pairing between individual Vδ and Vγ chains on the T cells. The TCR usage described for γδ T cells was that which was present at the time of functional assays. Our ability to digitally quantify the presence of mRNA species enabled us to determine that the propagated CAR$^+$ T cells expressed a polyclonal population of TCRγδ chains.
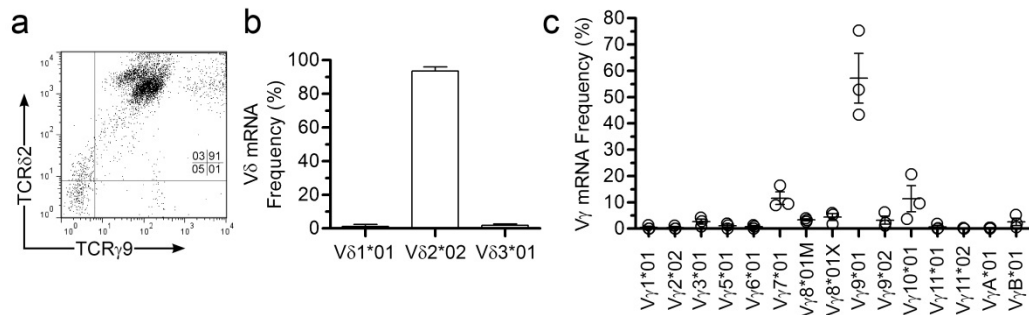
**Figure 21. Distribution of Vδ and Vγ in γδ T cells Expanded on Aminobisphosphonate.** (**a**) Representative flow cytometry plot from T cells following 36 days of numeric expansion with Zol. (**b**) Vδ and (**c**) Vγ allele mRNA expression in Zol-expanded T cells. Data are mean ± SD (n = 3). Quadrant frequencies of flow plot are displayed. **This work was originally published in *Molecular Therapy*.** Deniger, D. C., K. Switzer, T. Mi, S. Maiti, L. Hurton, H. Singh, H. Huls, S. Olivares, D. A. Lee, R. E. Champlin, and L. J. Cooper. 2013. Bispecific T-cells Expressing Polyclonal Repertoire of Endogenous gammadelta T-cell Receptors and Introduced CD19-specific Chimeric Antigen Receptor. Mol Ther. 21(3): 638-647.(311) © **Nature Publishing Group**
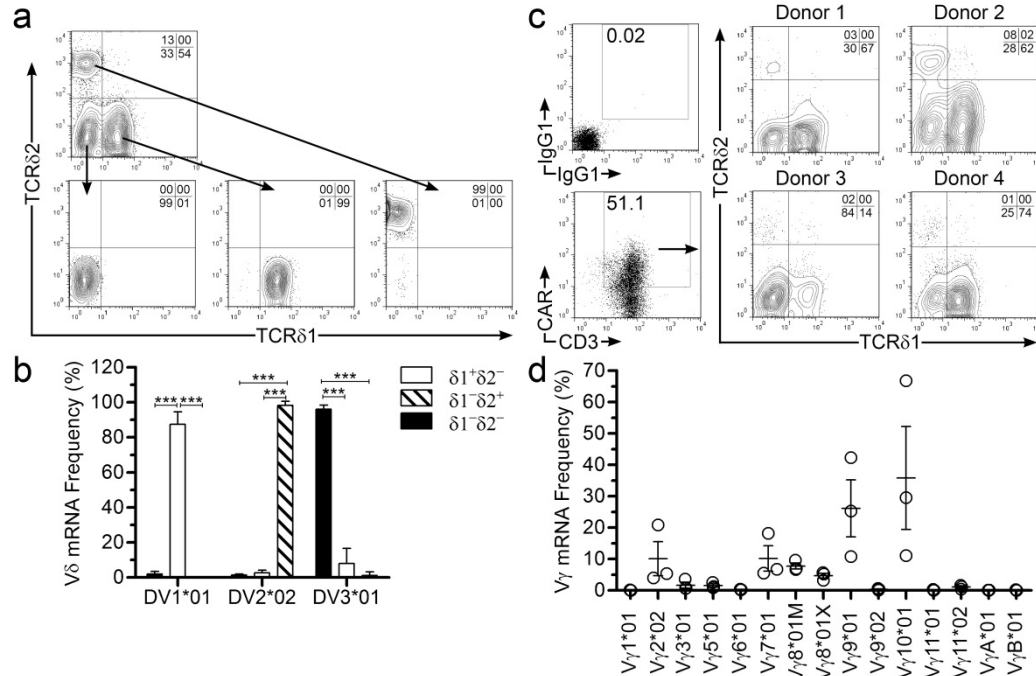
**Figure 22. Distribution of Vδ and Vγ in CAR⁺ γδ T cells.** (**a**) Representative FACS of Vδ populations (top) into Vδ1$^{neg}$Vδ2$^{neg}$ (left), Vδ1$^+$Vδ2$^{neg}$ (middle), and Vδ1$^{neg}$Vδ2$^+$ (right) populations and (**b**) Vδ allele mRNA expression in sorted T cells. (**c**) Vδ1$^{neg}$Vδ2$^{neg}$, Vδ1$^+$Vδ2$^{neg}$, and Vδ1$^{neg}$Vδ2$^+$ frequencies in gated CAR⁺ γδ T-cell populations from four donors. (**d**) Vγ allele mRNA expression in CAR⁺ γδ T cells. Data are mean ± SD (n = 3). Quadrant percentages of flow plots are in upper right corner. **This work was originally published in *Molecular Therapy*** Deniger, D. C., K. Switzer, T. Mi, S. Maiti, L. Hurton, H. Singh, H. Huls, S. Olivares, D. A. Lee, R. E. Champlin, and L. J. Cooper. 2013. Bispecific T-cells Expressing Polyclonal Repertoire of Endogenous gammadelta T-cell Receptors and Introduced CD19-specific Chimeric Antigen Receptor. Mol Ther. 21(3): 638-647.(311) © **Nature Publishing Group**

III.C.4. T cells Produced Pro-inflammatory Cytokines in Response to Stimulation through Endogenous TCRγδ and Introduced CAR

The functional activity of the CAR$^+$ T cells was assessed by activation with leukocyte activation cocktail (LAC), which was comprised of PMA and Ionomycin. LAC mimics activation through TCR by simulating protein kinase C and increasing intracellular $Ca^{2+}$ to activate phospholipase C (PLC). Measurement of secreted and intracellular cytokines (in the presence of the inhibitor GolgiPlug, which contains Brefeldin A) were performed on genetically modified T cells with and without LAC (**Figure 23a and 23b**). A broad range of cytokines were produced by γδ T cells, with the highest expression of IFNγ, TNFα, and chemokines MIP-1α, MIP-1β, and RANTES (**Figure 23b**). Interleukin-17 (IL17) has been shown to be important for anti-tumor efficacy of γδ T cells and this cytokine was secreted by CAR$^+$ γδ T cells. These results suggest that TCRγδ can be activated to produce cytokines that could promote inflammation within the tumor. Next, CAR-specific cytokine production was assessed by activation using the murine T-cell lymphoma line EL4 and a genetically modified derivative to enforce expression of human CD19. Both TNFα and IFNγ were produced by CAR$^+$ γδ T cells in response to CD19 (**Figure 23c**). A less diverse repertoire of cytokines was secreted following CAR stimulation when compared with stimulation of TCRγδ, but IFNγ, TNFα, MIP-1α, MIP-1β, and RANTES were all increased in response to activation through CAR (**Figure 23d**). In aggregate, pro-inflammatory cytokines were upregulated by bi-specific CAR$^+$ γδ T cells through their TCR and CAR.
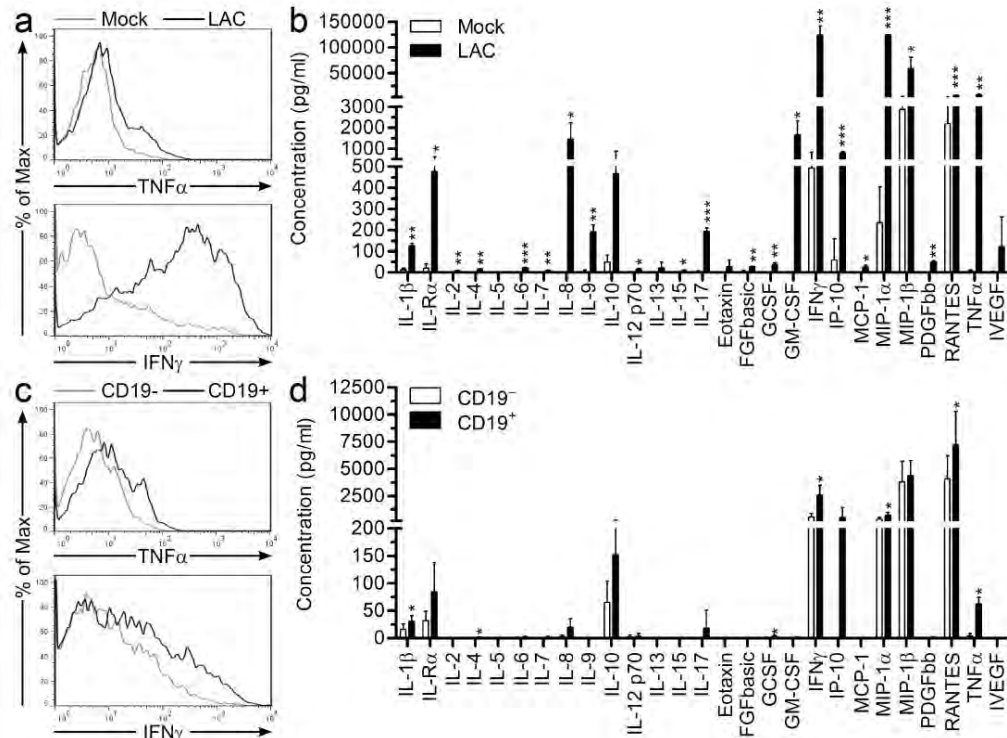
**Figure 23. Bi-specific γδ T cells Produce Pro-inflammatory Cytokines when Endogenous TCR and Introduced CAR are Stimulated.** (**a**) CAR$^+$ γδ T cells at Day 35 of co-culture on aAPC were stimulated for 4 hours with a mock cocktail (media alone) or Leukocyte Activation Cocktail (LAC, PMA/Ionomycin) to induce TCR stimulation and then analyzed by flow cytometry. CAR$^+$ T cells were gated and tumor necrosis factor-α (TNFα, top) and interferon-γ (IFNγ, bottom) production is shown. (**b**) Luminex array (27-Plex) of cytokines secreted by CAR$^+$ γδ T cells in conditions described in (**a**). (**c**) Similar to (**a**) except that EL4-CD19$^{neg}$ and EL4-CD19$^+$ were used instead of Mock/LAC. (**d**) Same as (**b**) but with EL4-CD19$^{neg}$ and EL4-CD19$^+$ targets. Student's t-test for statistical analysis between (**b**) Mock and LAC and (**d**) EL4-CD19$^{neg}$ and EL4-CD19$^+$ where *p<0.05, **p<0.01, and ***p<0.001. Data are representative of four donors for (**a**) and (**c**) and mean ± SD (n = 3) for (**b**) and (**d**). **This work was originally published in *Molecular Therapy*.** Deniger, D. C., K. Switzer, T. Mi, S. Maiti, L. Hurton, H. Singh, H. Huls, S. Olivares, D. A. Lee, R. E. Champlin, and L. J. Cooper. 2013. Bispecific T-cells Expressing Polyclonal Repertoire of Endogenous gammadelta T-cell Receptors and Introduced CD19-specific Chimeric Antigen Receptor. Mol Ther. 21(3): 638-647.(311) © **Nature Publishing Group**

III.C.5. CAR$^+$ γδ T cells Exhibit Enhanced Anti-tumor Effects against CD19$^+$

Targets *in vitro*

It was anticipated that γδ T cells would display endogenous cytotoxicity to leukemia cells. Therefore, γδ T cells without CAR were numerically expanded on aAPC in order to test their anti-leukemia activity. Human CD19$^+$ B-ALL cell lines (REH, Kasumi2, and Daudi genetically modified to express β2M) were lysed by CAR$^{neg}$ γδ T cells while primary, healthy CD19$^+$ B cells were not killed by the same effectors (**Figure 24a**). However, not all B-ALL cell lines were susceptible to efficient lysis by CAR$^{neg}$ γδ T cells. In particular, EL4 and NALM6 cells were largely resistant to cytolysis by γδ T cells. Thus, the ability of the CD19-specific CAR to amplify the inherent anti-tumor activity of γδ T cells was investigated. Enforced expression of CD19 on the surface of EL4 cells improved targeting and killing of this cell line by CAR$^+$ γδ T cells at significantly higher (p = 0.0001) levels compared with the parental CD19$^{neg}$ EL4 cell line (**Figure 24b**). Similarly, CAR$^+$ γδ T cells exhibited improved ability (p = 0.001) to kill CD19$^+$ NALM6 cells compared with CAR$^{neg}$ γδ T cells (**Figure 24c**). In summary, the introduced CAR enhanced the specific killing capability of genetically modified γδ T cells.
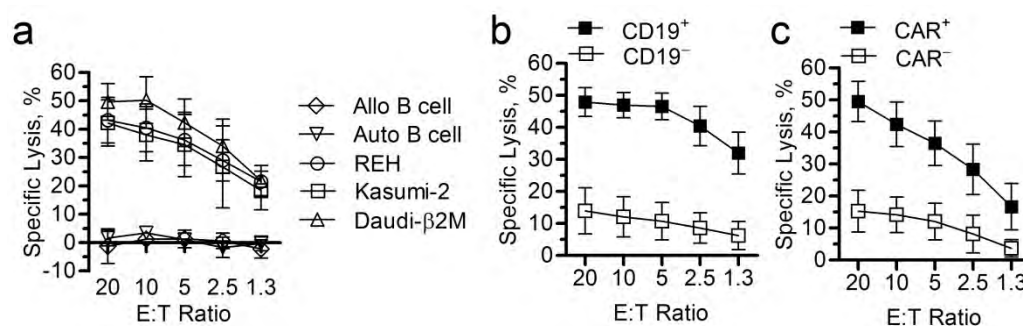
**Figure 24. Specific lysis of CD19$^+$ Tumor Cell Lines by CAR$^+$ γδ T cells.** (**a**) Standard 4-hour CRA of (**a**) CAR$^{neg}$ γδ T cells against CD19$^+$ B-ALL cell lines (REH, Kasumi2, and Daudi-β2M) or primary CD19$^+$ B cells from autologous (Auto) or allogeneic (Allo) donors, (**b**) CAR$^+$ γδ T cells against EL4-CD19$^{neg}$ (open squares) and EL4-CD19$^+$ (closed squares) tumor cells, and (**c**) CAR$^{neg}$ γδ T cells (open squares) and CAR$^+$ γδ T cells (closed squares) against CD19$^+$ NALM6 tumor cells. Data are mean ± SD from four healthy donors (average of triplicate measurements for each donor) that were pooled from two independent experiments. **This work was originally published in *Molecular Therapy*.** Deniger, D. C., K. Switzer, T. Mi, S. Maiti, L. Hurton, H. Singh, H. Huls, S. Olivares, D. A. Lee, R. E. Champlin, and L. J. Cooper. 2013. Bispecific T-cells Expressing Polyclonal Repertoire of Endogenous gammadelta T-cell Receptors and Introduced CD19-specific Chimeric Antigen Receptor. Mol Ther. 21(3): 638-647.(311) © **Nature Publishing Group**

### III.C.6. CAR$^+$ γδ T cells can Target CD19$^+$ Tumor *in vivo*

The ability of electroporated and propagated γδ T cells to target CD19$^+$ tumor was then investigated *in vivo*. NALM6 is an aggressive CD19$^+$ B-cell leukemia model and immunocompromised mice engrafted with $10^5$ NALM6 are moribund in 20 to 25 days when untreated. Control of disseminated NALM6 tumor *in vivo* is dependent on the infused T cells homing to tumor and activating cytolytic machinery in the tumor microenvironment. After adoptive immunotherapy, the burden of tumor was significantly decreased in mice receiving CAR$^+$ γδ T cells (Donor#4 from **Figure 22c**) compared to untreated mice (**Figure 25**). Mice in treatment group receiving CAR$^+$ T cells displayed fewer characteristics of the untreated and thus unwell mice, which included lethargy, ruffled coat, temporary hind limb paralysis, and difficulty entering and exiting anesthesia at late stages of the experiment. A uniform date for euthanasia was chosen to measure the anti-tumor effect based on flow cytometry for NALM6 in lymphoid tissue. There was significant anti-tumor activity by the CAR$^+$ γδ T cell as measured by bioluminescent imaging (BLI) of NALM6-eGFP-*ffLuc* (**Figure 25b**) as exemplified at 22 days after injection of tumor (**Figure 25c**). Non-invasive imaging was corroborated by analysis of presence of tumor cells at necroscopy. Mice that received CAR$^+$ γδ T cells exhibited significant reductions in tumor burden (CD19$^+$eGFP$^+$) in the bone marrow, spleen, and peripheral blood (**Figure 25d and 25e**). These data reveal that polyclonal CAR$^+$ γδ T cells exhibit therapeutic activity *in vivo*.
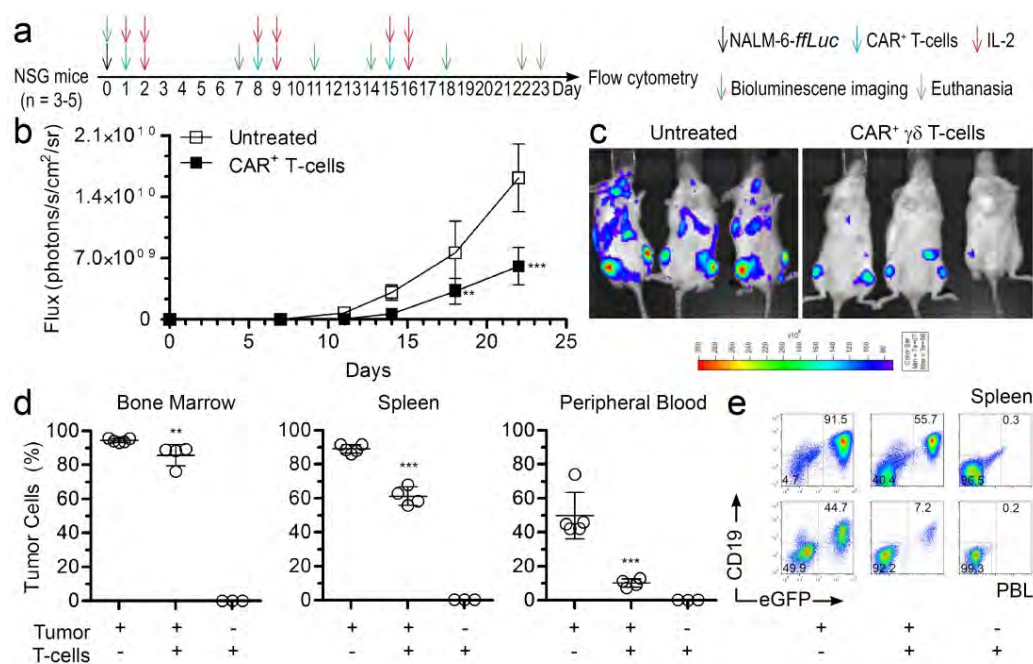
**Figure 25.** *In vivo* **Anti-tumor Activity of CAR⁺ γδ T cells.** (**a**) Schematic of experiment. (**b**) BLI derived from eGFP⁺*ffLuc*⁺CD19⁺ NALM-6 tumor and (**c**) representative images of mice at day 22. (**d**) Post-mortem analysis of tissues and blood where tumor cells (CD19⁺eGFP⁺) were detected by flow cytometry. (**e**) Representative flow plots from (**d**). Data are mean ± SD (n = 3 to 5 mice per group, representative of two independent experiments) and gating frequencies in (**e**) are displayed. The percentage of tumor cells is derived from detecting CD19⁺eGFP⁺ NALM-6 by flow cytometry from post-mortem samples. Statistics performed with (**b**) two-way ANOVA with Bonferroni's post-tests and (**d**) Student's t-test between treated and untreated mice. **p<0.01 and ***p<0.001 **This work was originally published in *Molecular Therapy*.** Deniger, D. C., K. Switzer, T. Mi, S. Maiti, L. Hurton, H. Singh, H. Huls, S. Olivares, D. A. Lee, R. E. Champlin, and L. J. Cooper. 2013. Bispecific T-cells Expressing Polyclonal Repertoire of Endogenous gammadelta T-cell Receptors and Introduced CD19-specific Chimeric Antigen Receptor. Mol Ther. 21(3): 638-647.(311) © **Nature Publishing Group**

## III.D. Discussion

III.D.1. Polyclonal Bi-specific T cells for Immunotherapy

We established that introduction of a $2^{nd}$ generation CAR could (i) drive the numeric expansion of T cells independent of usage of TCR$\gamma\delta$ chains and (ii) augment the lytic potential of $CD19^+$ tumors by $\gamma\delta$ T cells. Propagating bi-specific $CAR^+$ T cells with a broad diversity of TCR$\gamma\delta$ chains is desirable based on their therapeutic potential. Indeed, $\gamma\delta$ T cells other than those expressing V$\gamma$9V$\delta$2 have been generated from PBMC using TCR$\gamma\delta$-specific and CD3-specific mAbs.[313-315] These prior approaches did not comprehensively measure TCR$\gamma\delta$ isotype expression nor did they yield V$\delta$1 and V$\delta$3 at frequencies as high as seen in this study. The V$\gamma$2 TCR chain was detected on our T cells, which has been described to pair with V$\delta$2, and these T cells can have antigen presentation capabilities.[166] Our $CAR^+$ $\gamma\delta$ T cells expressed molecules consistent with antigen presentation, *e.g.*, CD86, CD137L, and HLA-DR (data not shown), and V$\gamma$9V$\delta$2 cells have served as aAPC for $\alpha\beta$ T cells.[298] Future experiments will investigate if our polyclonal $CAR^+$ $\gamma\delta$ T cells also have an ability to serve as aAPC. Also present were T-cell sub-populations expressing V$\gamma$7, and V$\gamma$8, and V$\gamma$10, where the first two chains have been associated with intestinal intraepithelial lymphocytes (iIEL)[316, 317] and the latter chain's functional significance is not yet apparent. In all, our approach is the first to report expansion of $CAR^+$ T cells that maintained a polyclonal TCR$\gamma\delta$ expression.

III.D.2. Changes Observed in Vδ Populations Following Expansion on aAPC

The repertoire of TCRγδ chains employed by CAR$^+$ T cells was similar to the initial pool of γδ T cells in PBMC with two exceptions. We noted an increase in Vδ3 usage, but this may be advantageous as it is associated with specificity for viruses that could offer enhanced immune responses to viral infections in immunocompromised patients receiving therapy.(165) A decrease in Vγ9Vδ2 usage was also observed compared to the starting frequency of this TCR in PBMC, but this could potentially be increased by priming aAPC with Zol to increase Vγ9Vδ2 ligand expression in the co-culture. Whether this loss of Vγ9Vδ2 TCR expression was due to preferential activation induced cell death or selective out-growth of T cells expressing Vδ1 and Vδ3 TCR is not known. Nonetheless, Vγ9Vδ2 chains were still present in the final T-cell cultures indicating that aminobisphosphonate therapy could drive expansion of this subset of T cells after administration.

III.D.3. Improvements upon CAR Expression on γδ T cells

Recombinant retroviruses have been previously employed to achieve stable expression of CARs in γδ T cells, but this required using an aminobisphosphonate to achieve numeric expansion of T cells before transduction.(175, 318) We now demonstrate propagation of T cells after, rather than before, gene transfer using SB-mediated transposition results in a

polyclonal population of bi-specific γδ T cells capable of CAR-mediated (i) production and secretion of pro-inflammatory cytokines in response to CD19, (ii) enhanced lysis of CD19$^+$ tumor targets, and (iii) *in vivo* anti-tumor activity against a CD19$^+$ tumor. The ability of these T cells to exhibit effector functions was not correlated to a particular Vδ or Vγ usage as cells with different Vδ TCR frequencies (**Figure 22c**) produced the same cytokines (**Figure 23**) and displayed similar cytolysis of CD19$^+$ targets (**Figure 24b**). We noted that frequency of CAR expression was more variable on γδ T cells compared with αβ T cells. This was likely due to an endogenous ability of K562 cells to sustain proliferation of γδ T cells independent of CAR. Nevertheless, adoptive transfer of γδ T cells of which 60% expressed CAR could still yield the same *in vitro* lytic ability as 98% CAR$^+$ γδ T cells (**Figure 26**). This indicated that (i) CAR$^+$ γδ T cells are potent tumor killers and (ii) >90% CAR expression may not be a critically limiting parameter for predicting therapeutic efficacy. Nonetheless, we are undertaking improvements to increase the expression of CAR on propagated γδ T cells. Furthermore, the chimeric signaling molecules in the CAR endodomain could be specifically designed to enhance triggering of γδ T cells. For example, γδ T cells can be activated through FcγRIIIA (CD16) in the TCR complex,(319) which raises the possibility that signaling through chimeric FcRγ (as compared with CD3ζ in our current design) in a CAR endodomain may improve activation. However, CD16 was not detected on CAR$^+$ γδ T cells in this study (data not shown). Since clinical responses against CD19$^+$ lymphocytic leukemia have been achieved with T cells expressing a CAR that signaled

111

through 4-1BB (CD137) endodomain,(7, 32) another option is to swap CD28 for

CD137 for activation of γδ T cells.
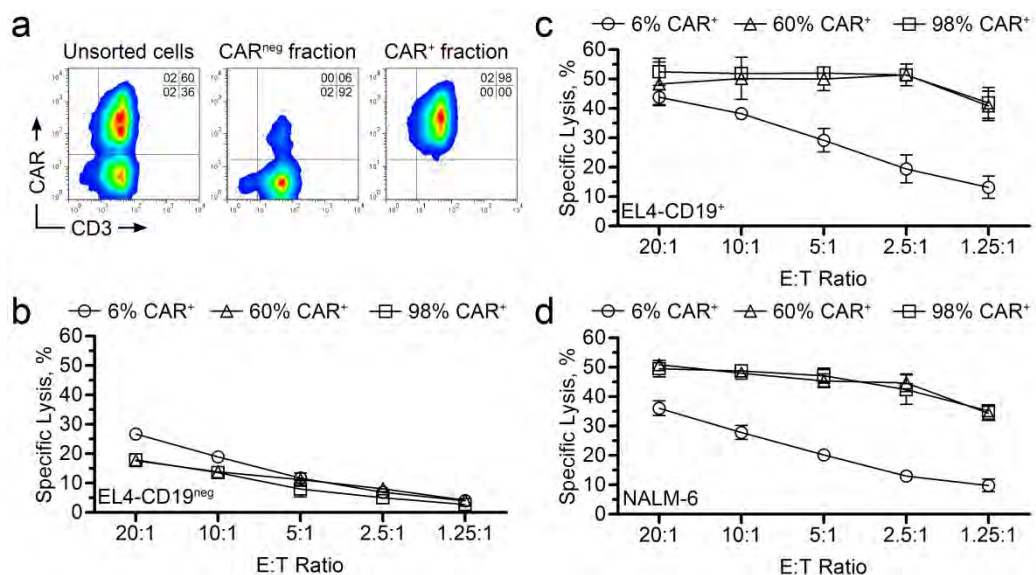
**Figure 26. Specific Lysis of CD19$^+$ Tumor Cell Lines by CAR$^+$, CAR$^{++}$, and CAR$^{+++}$ γδ T cells.** (**a**) Phenotype of T cells at day 19 of co-culture either unsorted (left) or from CAR sorting at day 15 where CAR$^{neg}$ and CAR$^+$ fractions are displayed in the middle and right, respectively. Four-hour CRA (Day 19 of co-culture on aAPC) of γδ T cells genetically modified to enforce expression of CD19-specific CAR with 6% (CAR$^+$, circles), 60% (CAR$^{++}$, triangles), and 98% (CAR$^{+++}$, squares) expression of CAR targeting (**b**) EL4-CD19$^{neg}$, (**c**) EL4-CD19$^+$, and (**d**) CD19$^+$ NALM-6 tumor cells. Data are mean ± SD (n = 3). Quadrant frequencies of flow plots are displayed. **This work was originally published in _Molecular Therapy_.** Deniger, D. C., K. Switzer, T. Mi, S. Maiti, L. Hurton, H. Singh, H. Huls, S. Olivares, D. A. Lee, R. E. Champlin, and L. J. Cooper. 2013. Bispecific T-cells Expressing Polyclonal Repertoire of Endogenous gammadelta T-cell Receptors and Introduced CD19-specific Chimeric Antigen Receptor. Mol Ther. 21(3): 638-647.(311) © **Nature Publishing Group**

III.D.4. Improvements on Type of γδ T cell used for CAR Immunotherapy

In addition to improving CAR expression on γδ T cells, the type of γδ T cell arising after electroporation with SB system and propagation on aAPC could be manipulated to further improve anti-tumor activity. For instance, some γδ T cells were observed to secrete IL17, a pro-inflammatory cytokine that has potent, yet context-dependent, anti-tumor effects.(320-324) IL17 producing lineages of T cells can be mutually exclusive from those that secrete IFNγ.(325) Inducible co-stimulator of T cells (ICOS) leads to IL17 polarization in CD4$^+$ T cells and CD28 co-stimulation overcame this effect to dictate that CD4$^+$ T cells now produce IFNγ.(326) CD86 is one of the co-stimulatory molecules on our aAPC and the majority of CAR$^+$ γδ T cells secrete IFNγ in response to CD19 with diminished production of IL17. Furthermore, the CAR contains a chimeric CD28 endodomain which may contribute to IFNγ polarization in genetically modified T cells. Substitution of chimeric CD28 for ICOS in the CAR and replacement of CD86 on the aAPC with ICOS-ligand (ICOSL) could potentially reverse the polarization to IL17. Given that we can propagate CAR$^+$ γδ T cells on aAPC we are prepared to design aAPC to evaluate whether we can skew the cytokine profile to reflect the propagation of desired T-cell subsets.

III.D.5. Clinical Significance of Bi-specific T cells

The human application of CAR$^+$ γδ T cells is appealing given their inherent potential for anti-tumor effects and their apparent lack of alloreactivity.(304) The CAR, SB system, and aAPC are all already in use in our clinical trials. Therefore, we plan to modify our manufacturing scheme in compliance with current good manufacturing practice to generate bi-specific CAR$^+$ γδ T cells. Our data provides a clinically-appealing approach to numerically expand and manipulate CAR$^+$ T cells with multiple Vγ and Vδ pairings enabling clinical trials to evaluate their therapeutic potential."

**This work was adapted from published work in *Molecular Therapy*.** Deniger, D. C., K. Switzer, T. Mi, S. Maiti, L. Hurton, H. Singh, H. Huls, S. Olivares, D. A. Lee, R. E. Champlin, and L. J. Cooper. 2013. Bispecific T-cells Expressing Polyclonal Repertoire of Endogenous gammadelta T-cell Receptors and Introduced CD19-specific Chimeric Antigen Receptor. Mol Ther. 21(3): 638-647.(311) © **Nature Publishing Group**

## **CHAPTER IV**

**Artificial Antigen Presenting Cells Propagate Polyclonal Gamma Delta T cells with**

**Broad Anti-tumor Activity**

### **IV.A. Hypothesis and Rationale**

The *hypothesis* of this chapter is that aAPC will expand polyclonal γδ T cells that will have broad anti-tumor immunity. The *rationale* for this chapter is that (i) CAR$^{neg}$ polyclonal γδ T cells proliferated in parallel to CAR$^{+}$ γδ T cells described in **Chapter III** on aAPC, (ii) no current expansion protocols exist for polyclonal γδ T cells for the clinic, (iii) aAPC are currently in clinical trials and are available as a master cell bank in the manufacturing facility at MD Anderson, (iv) γδ T cells expressing Vδ1 are correlated with long-term remissions in cancer therapy but have not been directly infused as an adoptive immunotherapy, (v) γδ T cells expressing Vδ2 have shown anti-tumor effects as direct adoptive immunotherapies, (vi) γδ T cells expressing Vδ3 have not been described to have direct anti-tumor immunity leaving a gap in the field of knowledge, and (vii) a polyclonal approach to γδ T cell immunotherapy could target multiple ligands on the tumor through a diverse repertoire of TCRγδ. Therefore, development of an expansion protocol to generate clinically-relevant numbers of polyclonal γδ T cells would have implications as both cancer immunotherapies and for immunologists studying γδ T cells.

## IV.B. Introduction

Human γδ T cells exhibit inherent anti-tumor activity and hold promise for immunotherapy of cancer. They are distinguished by the heterodimeric pairing of γ and δ T-cell receptor (TCR) chains from the more prevalent αβ T cell lineage (~95% of circulating T cells), which are defined by TCRα/TCRβ heterodimers.(327) TCRαβ recognizes peptide complexed with MHC but TCRγδ ligands are recognized independent of MHC restriction.(141, 146, 152) Many of these ligands are present on cancer cells, thus raising the possibility that a culturing approach to propagating T cells that maintains a polyclonal repertoire of γδ TCRs may have appeal for human application.

γδ T cells represent 1% to 5% of the T-cell pool in peripheral blood, and many standard T cell expansion protocols are not applicable to γδ T cells.(314, 328) Proliferation of monoclonal γδ T cell populations (Vγ9Vδ2) can be sustained with aminobisphosphonates, e.g. Zol, and clinical trials investigating their anti-tumor efficacy have yielded objective responses treating both solid and hematological cancers.(175, 179, 301) However, this subset of γδ T cells was not curative as a stand-alone therapy.(318) Novel polyclonal γδ T cell expansion protocols are needed to improve upon these findings, but are lacking in clinically-relevant methods to expand multiple γδ T cell subsets in one cellular therapy product.

Since many ligands that signal through γδ TCR are unknown, we hypothesized that a tumor cell line may serve as a cellular substrate for activating these T cells and sustaining their proliferation. aAPC are used to stimulate CAR$^+$ T cell growth *ex vivo*

and are derived from K562 cells, a natural cytolytic target of $\gamma\delta$ T cells.(57, 280, 310, 329) As seen in **Chapter III**, CAR-modified $\gamma\delta$ T cells expanded on aAPC while expressing multiple TCR$\gamma\delta$ alleles and displayed enhanced cytolysis to antigen-positive tumors.(311) Moreover, $\gamma\delta$ T cells not expressing CAR were present in CAR$^+$ $\gamma\delta$ T cell cultures in high frequencies (**Figure 19a, bottom right panels**). Therefore, we hypothesized that $\gamma\delta$ T cells could expand on aAPC independent of CAR$^+$ T cells and that these $\gamma\delta$ T cells would maintain a polyclonal TCR$\gamma\delta$ repertoire. Given that the aAPC are available as a master-cell bank, these data provide a translational pathway for adapting $\gamma\delta$ T cells for human application. Thus, this could be the first time that polyclonal $\gamma\delta$ T cells could be used for cancer immunotherapy.

## IV.C. Results

IV.C.1. Propagation of $\gamma\delta$ T cells on aAPC

As seen in **Chapter III**, aAPC clone#4 sustained the proliferation of $\gamma\delta$ T cells in cultures containing CD19-specific CAR$^+$ $\gamma\delta$ T cells.(311) To assess whether $\gamma\delta$ T cells could numerically expand on aAPC without expression of CAR, quiescent $\gamma\delta$ T cells were isolated from peripheral blood and stimulated by recursive additions of $\gamma$-irradiated aAPC clone#4 in presence of IL2 and IL21 (**Figure 27a**). It was observed that $\gamma\delta$ T cells represented a small fraction of PBMC (3.2% ± 1.2%; mean ± SD; n = 4), but after 22 days of co-culture on aAPC the cultures contained a homogeneous population of $\gamma\delta$ T cells (97.9% ± 0.6%) as assessed by co-expression of CD3 and

TCRγδ (**Figure 27b**). Cultures yielded $>10^9$ γδ T cells from $<10^6$ total cells in three weeks of co-culture (**Figure 27c**), which represented a $4.9 \times 10^3 \pm 1.7 \times 10^3$ fold increase over a 22-day culture period. Although γδ T cells were rare in peripheral blood, they were readily sorted then expanded on aAPC to sufficient numbers for experiments and potential clinical application.
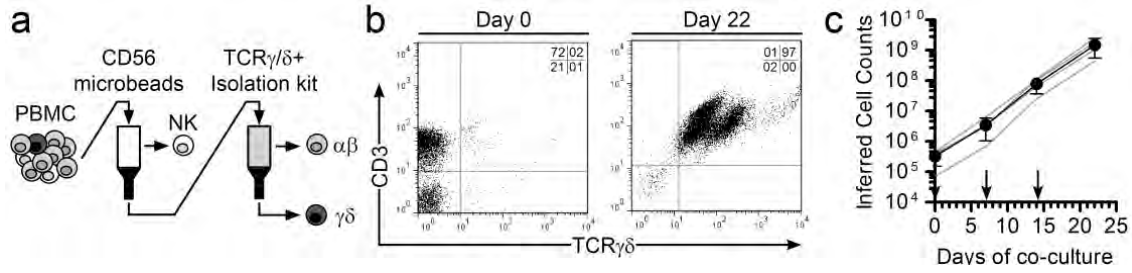
**Figure 27. Sustained Proliferation of γδ T cells on aAPC and IL2/21.** (**a**) Schematic of experimental design where NK cells are in open shapes, αβ T cells are in light gray shapes, and γδ T cells are in dark gray shapes. Columns represent paramagnetic isolation. (**b**) Expression by flow cytometry of CD3 (y-axis) and TCRγδ (x-axis) in PBMC prior to isolation of γδ T cells isolation (Day 0) and after 22 days of co-culture on aAPC/IL2/IL21. One representative donor is shown and quadrant gate frequencies are displayed in the upper right corners of flow plots. (**c**) Total inferred cell counts of viable cells during co-culture period. Black lines are mean ± SD from 4 healthy donors, gray lines are individual donors, and arrows represent addition of γ-irradiated aAPC.

IV.C.2. Roles for Co-stimulation and Cytokine Support in γδ T cell Proliferation on aAPC

The mechanism of γδ T cell proliferation on aAPC was unknown. Addition of cytokines and co-stimulation by aAPC were likely candidates for supporting growth of on aAPC. In order to assess which surface molecules on the clone#4 aAPC (membrane-bound IL15 (mIL15), CD86, and CD137L) were important for γδ T cell expansion with IL2 and IL21, parental K562 cells were genetically modified to express (i) mIL15 (cloneA6), (ii) mIL15 and CD86 (clone A3), or (iii) mIL15 and CD137L (clone D4) and were subcloned for uniform transgene expression (**Figure 28a**). Co-cultures with exogenous IL2 and IL21 were initiated with γδ T cells and γ-irradiated (i) parental K562 cells, (ii) clone A6 aAPC, (iii) clone A3 aAPC, (iv) clone D4 aAPC (**Figure 28b**), or (v) clone#4 aAPC (**Figure 8 middle panels**) in parallel with T cells receiving cytokines only. IL2 and IL21 in combination sustained limited γδ T cell proliferation, which was increased when K562 cells were added to co-cultures. Slightly less expansion was observed when either mIL15 or mIL15 and CD86 were added to K562 cells. However, significantly higher γδ T cell propagation was only observed with mIL15$^+$CD137L$^+$ and mIL15$^+$CD86$^+$CD137L$^+$ aAPC over IL2 and IL21 alone. After establishing that co-stimulation on aAPC was necessary for γδ T cell proliferation, IL2 and IL21 were added separately or in combination to assess their contribution to growth on clone#4 aAPC. No γδ T cell expansion was observed when both IL2 and IL21 were removed from co-cultures, addition of IL2 alone resulted in more proliferation than IL21 alone, and combination of both IL2 and IL21 displayed additive growth of γδ T cells (**Figure 28c**).

This validated our approach to use both IL2 and IL21 for maximum γδ T cell yield following co-culture on clone#4 aAPC and strongly suggested that both aAPC co-stimulation and cytokine support were critical for maximum γδ T cell proliferation *ex vivo*.
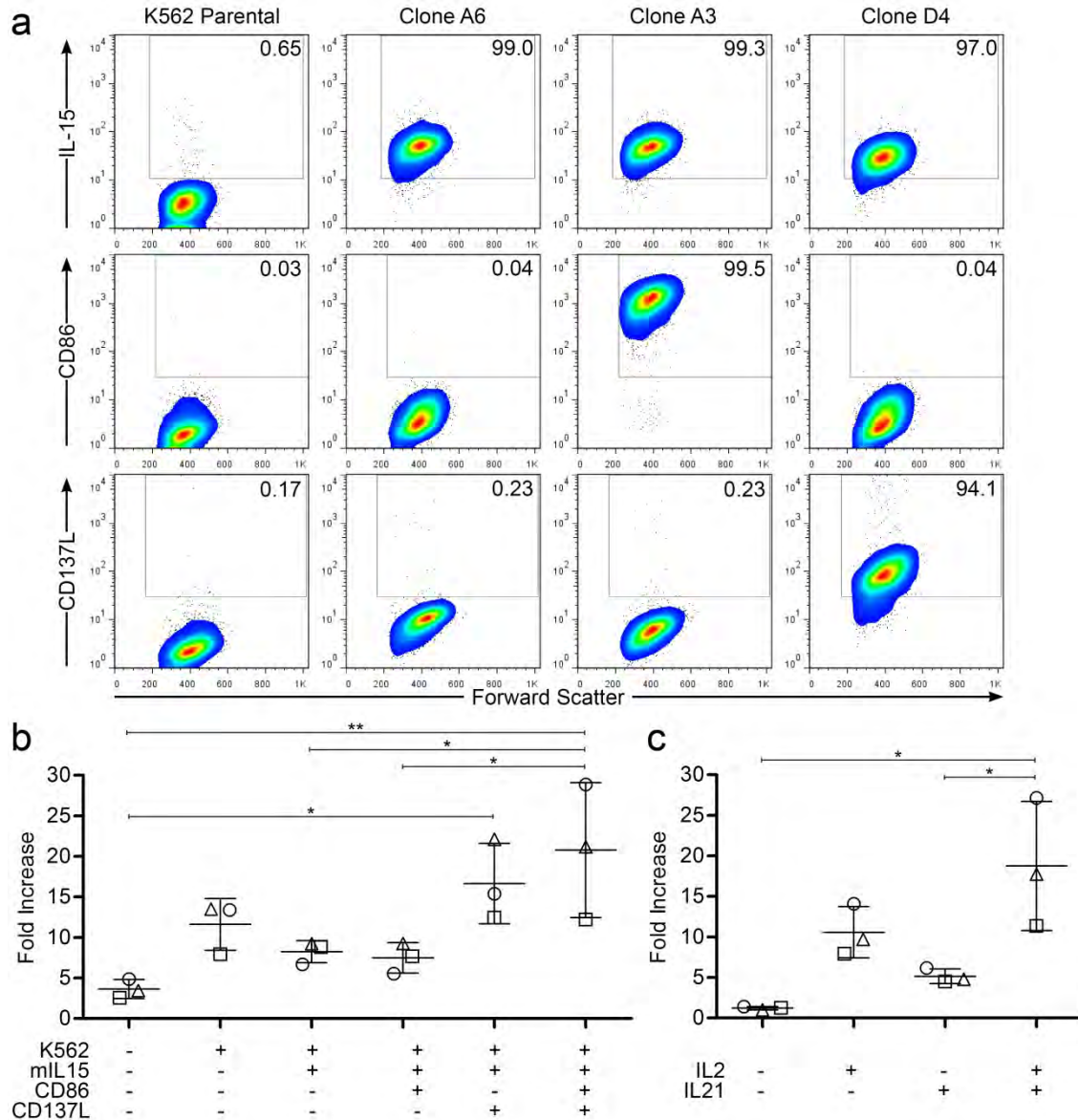
**Figure 28. Co-stimulation and Cytokine Requirements for γδ T cell Expansion on aAPC *ex vivo*.** (**a**) Surface phenotype of aAPC expressing single co-stimulatory molecules with membrane-bound IL15 (mIL15). (**b**) γδ T cell proliferation was measured after 10 days of growth with IL2 and IL21 on (i) no aAPC, (ii) parental K562 cells, (iii) mIL15$^+$ aAPC (clone A6), (iv) mIL15$^+$CD86$^+$ aAPC (clone A3), (v) mIL15$^+$CD137L$^+$ aAPC (clone D4), or (vi) clone#4 aAPC. All aAPC were γ-irradiated prior to co-culture. (**c**) Co-cultures were initiated with clone#4 aAPC and either (i) no cytokines, (ii) 50 U/mL IL2, (iii) 30 ng/mL IL21, or (iv) 50 U/mL IL2 and 30 ng/mL IL21. Fold changes were calculated relative to the input cell numbers. Two-way ANOVA with Bonferroni's post-tests was used for statistical analysis. *p<0.05 and **p<0.01

IV.C.3. UCB-derived γδ T cells Expansion on aAPC

Umbilical cord blood (UCB) is a source of γδ T cells with unique use for immunotherapy because they have limited immunological education and thus potential utility in allogeneic settings. Moreover, UCB-derived γδ T cells should have a younger phenotype and could (theoretically) have a longer range of responsiveness before anergizing or undergoing senescence. However, UCB has limited volumes and γδ T cells are a small fraction of an already limited resource. Fluorescence activated cell sorting (FACS) was used to isolate γδ T cells in order to maximize yields and purity of this valuable resource. Indeed, clone#4 aAPC induced substantial proliferation of γδ T cells derived from UCB (**Figure 29a**). After 35 days of co-culture on clone#4 with IL2 and IL21, there was a 10 million-fold increase in cell number as an average of $10^{11}$ UCB-derived γδ T cells (Range: $6\times10^9 - 3\times10^{11}$; n = 5) were propagated from just $10^4$ γδ T cells at the start of the culture. Because few cells were isolated ($10^4$ per donor), two more stimulations were performed for UCB compared to PBMC to highlight their potential for proliferating to clinically relevant numbers. As expected, γδ T cell cultures were pure as assessed by uniform expression of CD3 (**Figure 29b**) and TCRγδ (**Figure 29c**) without expression of TCRαβ (**Figure 29d**) or presence of $CD3^{neg}CD56^+$ NK cells (**Figure 29b**). Collectively, these data demonstrate that aAPC clone#4 when used with IL2 and IL21 could sustain the proliferation of γδ T cells *ex vivo* from limited starting populations.
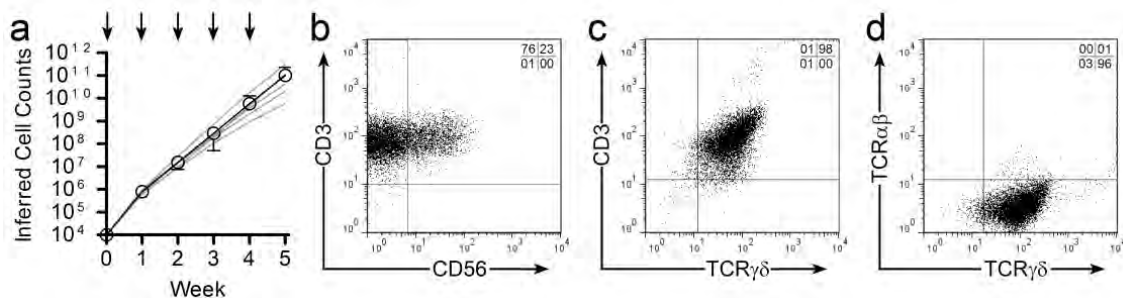
**Figure 29. Expansion of UCB-derived γδ T cells on aAPC.** γδ T cells were sorted by FACS following staining with CD3 and TCRγδ and were stimulated weekly with clone#4 aAPC, IL2, and IL21 (**a**) Total inferred cell numbers from co-cultures where black line represents mean ± SD (n = 5) and gray lines are individual donors. Arrows represent stimulations with aAPC. Expression of (**b**) CD3 (y-axis) and CD56 (x-axis), (**c**) CD3 (y-axis) and TCRγδ (x-axis), and (**d**) TCRαβ (y-axis) and TCRγδ (x-axis) of one representative donor by flow cytometry after 5 weeks of expansion on aAPC with IL2 and IL21. Quadrant frequencies are displayed in upper right corners.

IV.C.4. Frequency of γ and δ TCR Usage in aAPC-propagated γδ T cells

Previously, CAR⁺ γδ T cells expanded on clone#4 aAPC maintained polyclonal repertoire of TCRγ and TCRδ chains, and γδ T cells proliferating in parallel to CAR⁺ γδ T cells also maintained polyclonal TCRγδ distribution (**Chapter III**).(311) Whether the aAPC-expanded γδ T cells would do the same was of great interest, because if so then this would represent the first ever clinically-viable approach to expand multiple γδ T cells subsets in one cellular product for cancer therapy.

*IV.C.4.a. Vδ and Vγ mRNA Expression*

Now that it is established that γδ T cells can expand on aAPC independently of CAR⁺ T cells (**Figures 27, 28, and 29**), the TCR isotype variable (V) region repertoire was evaluated at the mRNA level by DTEA. As anticipated, mRNA species for all three Vδ alleles were identified (**Figure 30a**) and Vγ2, Vγ5, Vγ7, Vγ8 (two alleles), Vγ9, Vγ10, and Vγ11 mRNA species were co-expressed in the aAPC-expanded γδ T cells from PBMC (**Figure 30b**). Similar polyclonal TCR expression of Vδ (**Figure 30c**) and Vγ (**Figure 30d**) was observed in γδ T cells expanded from UCB with fewer Vδ2 cells, more Vγ2 and Vγ5 cells, and presence of Vγ3 cells not seen in PBMC. Thus, aAPC are able to repeatedly expand γδ T cells with polyclonal TCR repertoire from both PBMC and UCB.
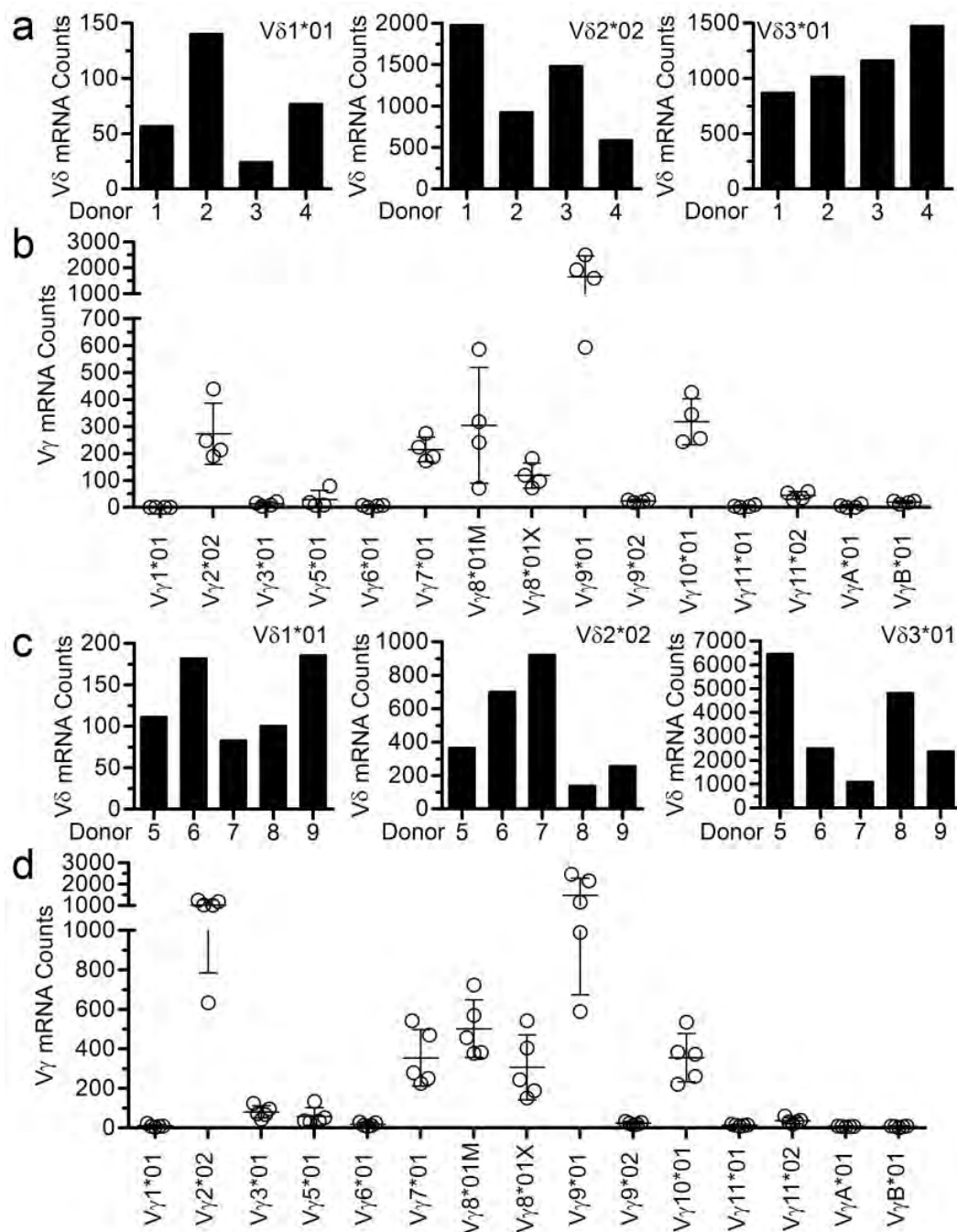
**Figure 30. Pattern of Vδ and Vγ mRNA Usage on aAPC-expanded γδ T cells.** Quantification of mRNA species coding for (**a**) Vδ1*01, Vδ2*02, and Vδ3*01 alleles from left to right, respectively, and (**b**) Vγ alleles in PBMC-derived γδ T cells by DTEA at day 22 of co-culture on aAPC/IL2/IL21. Each circle represents an individual donor's γδ T cells and lines show mean (horizontal) ± SD (vertical). Quantification of mRNA species coding for (**c**) Vδ and (**d**) Vγ alleles in UCB-derived γδ T cells by DTEA at day 34-35 of co-culture on aAPC/IL2/IL21 as described for PBMC. Numbers correlate with identification of PBMC (1-4) and UCB (5-9) donors described further in **Figure 31**.

127

*IV.C.4.b. TCRγδ Surface Protein Expression*

After establishing Vγ and Vδ mRNA expression from a number of different isotypes, surface expression of TCRγ and TCRδ was investigated. However, there are only 3 commercially available antibodies specific for individual TCRγδ isotypes, which are specific for TCRδ1, TCRδ2, and TCRγ9. As was seen in CAR$^+$ γδ T cells, aAPC-expanded γδ T cells from PBMC stained for all three Vδ populations (TCRδ1$^+$TCRδ2$^{neg}$, TCRδ1$^{neg}$TCRδ2$^+$, and TCRδ1$^{neg}$TCRδ2$^{neg}$), corroborating DTEA detection of Vδ1, Vδ2, and Vδ3 populations of γδ T cells, respectively (**Figure 31a**). Moreover, TCRδ expression frequencies followed the trend of TCRδ1>TCRδ3>TCRδ2, and most TCRδ2 chains paired with TCRγ9 (**Figure 31b**). Fewer TCRδ2 cells were seen in UCB-derived γδ T cells (**Figure 31c**) compared to PBMC-derived γδ T cells (**Figure 31a**), but UCB-derived γδ T cells followed the same TCRδ1>TCRδ3>TCRδ2, trend and most Vδ2 paired with Vγ9 as expected (**Figure 31d**). Analysis of other Vγ pairings with Vδ could not be performed because there are no other Vγ-specific commercially antibodies available. Thus, aAPC-expanded γδ T cells were polyclonal at both mRNA and protein levels, and this protocol therefore represents the first clinically-relevant expansion approach of polyclonal γδ T cells.
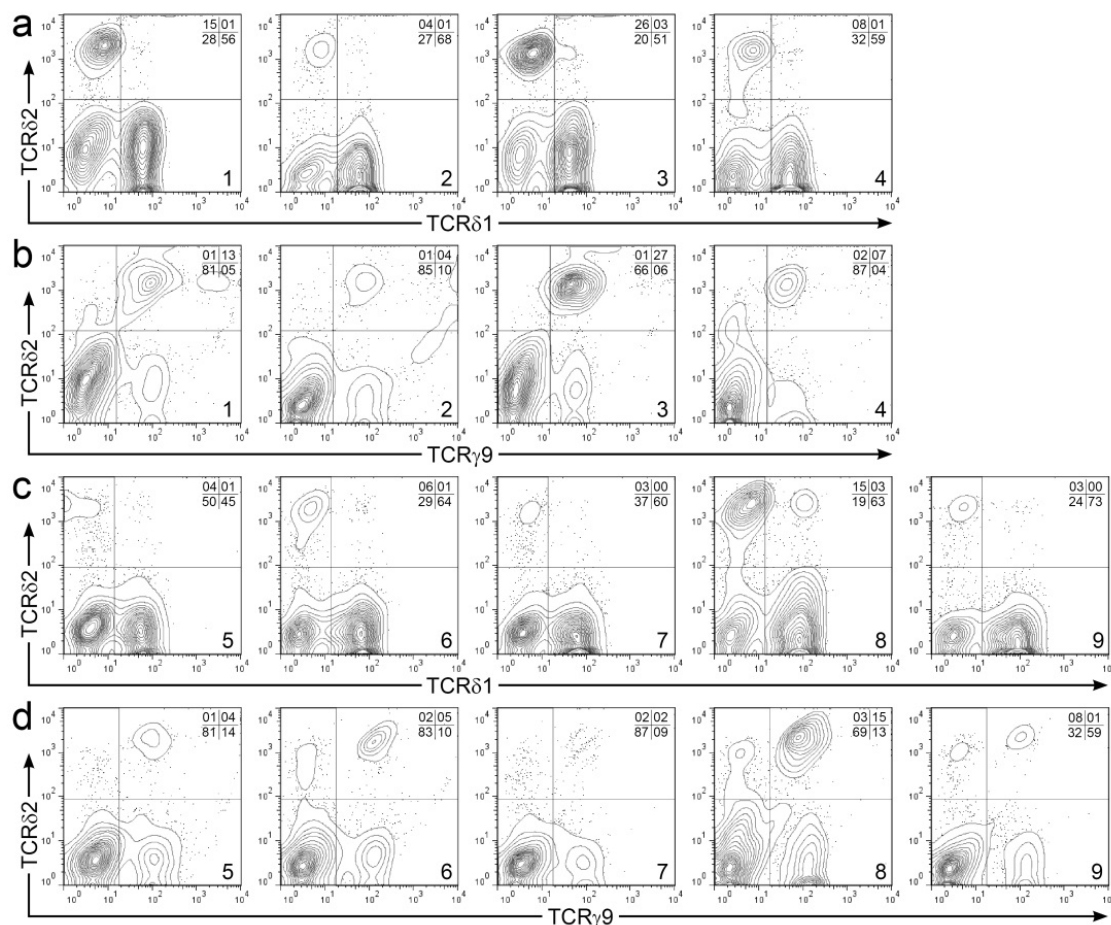
**Figure 31. TCRδ and TCRγ Isotype Surface Expression on aAPC-expanded γδ T cells.** Expression by flow cytometry of (**a**) TCRδ2 (y-axes) and TCRδ1 (x-axes) and (**b**) TCRδ2 (y-axes) and TCRγ9 (x-axes) in PBMC-derived γδ T cells at day 22 of co-culture on aAPC/IL2/IL21. Expression by flow cytometry of (**c**) TCRδ2 (y-axes) and TCRδ1 (x-axes) and (**d**) TCRδ2 (y-axes) and TCRγ9 (x-axes) in UCB-derived γδ T cells at day 35 of co-culture on aAPC/IL2/IL21. Numbers in lower right corners correlate with identification of PBMC (1-4) and UCB (5-9) donors also shown in **Figure 30** and quadrant frequencies are displayed in upper right corners.

*IV.C.4.c. Validation of Vδ3 Subset and Vδ Lineage Propagation*

Little is known about the Vδ3 lineage of γδ T cells and no reports have been made to date about their role in anti-tumor immunity. Because this study has implications for showing the first ever evidence that this subset can mediate anti-tumor effects, further validation that the TCRδ1$^{neg}$TCRδ2$^{neg}$ cells were, in fact, Vδ3 cells was warranted. Complicating this matter is the fact that no commercially available antibodies for TCRδ3. However, an indirect means was successfully used by combining FACS and DTEA. As there are only three Vδ populations in humans and there are antibodies to two of the isoforms, a combination of DTEA and FACS was used to in two ways to confirm the various populations. First, γδ T cells expanded in the presence of CAR$^+$ T cells (**Chapter III**) were sorted for TCRδ1$^+$TCRδ2$^{neg}$, TCRδ1$^{neg}$TCRδ2$^+$, and TCRδ1$^{neg}$TCRδ2$^{neg}$ γδ T cells populations by FACS and they expressed only Vδ1*01, Vδ2*02, and Vδ3*01 mRNA, respectively (**Figure 22a and 22b**).(311) The second approach directly applied the same techniques to γδ T cells expanded on aAPC as described in **Chapter IV** without CAR$^+$ T cells. Again, TCRδ1$^+$TCRδ2$^{neg}$, TCRδ1$^{neg}$TCRδ2$^+$, and TCRδ1$^{neg}$TCRδ2$^{neg}$ populations isolated by FACS consisted primarily of Vδ1*01, Vδ2*02, and Vδ3*01 mRNA, respectively, and were therefore denoted Vδ1, Vδ2, and Vδ3, respectively (**Figure 32a**). It is important to note that Vδ1*01 only resulted in ~150 mRNA counts whereas Vδ2*02 and Vδ3*01 ranged in the ~1000-2000 mRNA count range (**Figure 30**), so the purity as measured by mRNA counts appeared to have contaminating Vδ1 cells in Vδ2 and Vδ3 populations but these populations were minor contributors in the Vδ1 population as measured by flow

cytometry (**Figures 32d and 32e**). Furthermore, the FACS sorted Vδ1, Vδ2, and Vδ3 populations were expanded on clone#4 aAPC in the presence of exogenous IL2 and IL21 as separate co-cultures populations and even after 15 days of isolated growth the same Vδ mRNA signatures were observed suggesting the cells remained pure during propagation (data not shown). As expected, all three Vδ populations proliferated well on aAPC as separate populations (**Figure 32b**), where fold increase capability was ranked as Vδ1>Vδ3=Vδ2 although there were no statistically different differences (**Figure 32c**). Indeed, there are more Vδ1 cells in polyclonal populations (**Figures 30 and 31**), which may be due to a slight increase in their ability to proliferate on aAPC. Importantly, populations expressed the appropriate TCR alleles on the γδ T cell surface where Vδ1, Vδ2, and Vδ3 subsets were pure for TCRδ1$^+$TCRδ2$^{neg}$, TCRδ1$^{neg}$TCRδ2$^+$, and TCRδ1$^{neg}$TCRδ2$^{neg}$, respectively, after 15 days of isolated expansion on aAPC (**Figure 32d and 32e**). All separated Vδ subsets co-expressed CD3 and TCRγδ verifying that they were, in fact, γδ T cells (**Figures 34a and 34b**). Collectively, these results showed that (i) TCRδ1$^{neg}$TCRδ2$^{neg}$ γδ T cells contained the Vδ3 lineage, (ii) DTEA accurately measured Vδ mRNA, (iii) Vδ1, Vδ2, and Vδ3 lineages are stimulated by aAPC leading to their proliferation, and (iv) aAPC-expanded γδ T cells are truly polyclonal.
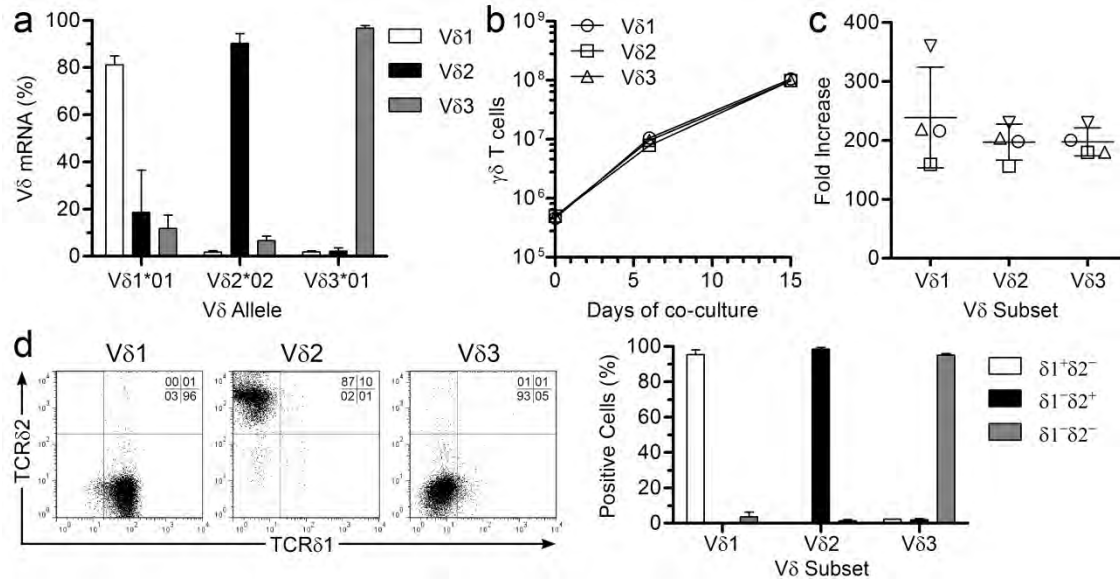
**Figure 32. Vδ Subset Separation, Propagation, and Resultant TCR Expression on Sorted T cells.** PBMC were sorted for γδ T cells with paramagnetic beads and were expanded for 2 weeks on aAPC/IL2/IL21. They were then sorted into three populations (Vδ1, Vδ2, and Vδ3) by FACS. Separated populations were stimulated for 2 weeks on aAPC/IL2/IL21. (**a**) DTEA detection of Vδ1*01, Vδ2*02, and Vδ3*01 mRNA species in Vδ1, Vδ2, and Vδ3 subsets following FACS purification. (**b**) Proliferation of Vδ lineages on aAPC as separated populations. (**c**) Fold increases of each Vδ population where each shape represents a different donor. (**d**) Representative flow cytometry plots of TCRδ1 (x-axes) and TCRδ2 (y-axes) expression in Vδ1, Vδ2, and Vδ3 subsets (from left to right). Quadrant frequencies are displayed in upper right corner. (**e**) Frequencies of TCRδ1+TCRδ2neg, TCRδ1negTCRδ2+, and TCRδ1negTCRδ2neg cells in Vδ1, Vδ2, and Vδ3 subsets. Data are mean ± SD (n = 3-4).

IV.C.5. Immunophenotype of γδ T cells Expanded on aAPC

Functional outcomes, e.g. memory formation, homing to tissues, and effector mechanism, can be predicted by the expression of lymphocyte-specific proteins on the T cell surface. Thus, a panel of markers was used to identify the immunophenotype of γδ T cells cultures first as a polyclonal population to be used as therapy and then as sorted Vδ populations to gain insight into lineage differences.

*IV.C.5.a. Immunophenotype of Polyclonal γδ T cell Population*

The ultimate goal for the clinic is to use a polyclonal population of T cells for immunotherapy in order to have a multivariate approach to cancer immunotherapy, so extensive phenotyping of the γδ T cell surfaces was performed as a mixed Vδ population. After 22 days of co-culture on aAPC, few αβ T cells (TCRαβ) and NK cells (CD3$^{neg}$CD56$^{+}$) were detected in the cultures where strong staining for γδ T cells (TCRγδ) was observed (**Figure 33a**). Most γδ T cells were CD4$^{neg}$CD8$^{neg}$, as expected,(286) but some CD8 and CD4 expression was observed (**Figure 33b**). These T cells were highly activated as measured by expression of CD38 and CD95. IL2 receptors (CD25; IL2Rα and CD122; IL2Rβ) were detected, but limited surface expression of IL7Rα (CD127) was identified. γδ T cells were not exhausted as evidenced by the absence of CD57 and PD1. Most cells expressed CD27 and CD28 co-stimulatory ligands and had a preference towards antigen-experienced (CD45RO) over naïve (CD45RA) characteristics. Homing to the skin, lymph nodes, and bone marrow

has potential as evidenced by CCR4, CXCR4/CLA, and CCR7/CD62L expression, respectively. In aggregate, the surface phenotypes of γδ T cells indicated that they were highly activated and antigen experienced with potential for memory formation and homing to tissues.
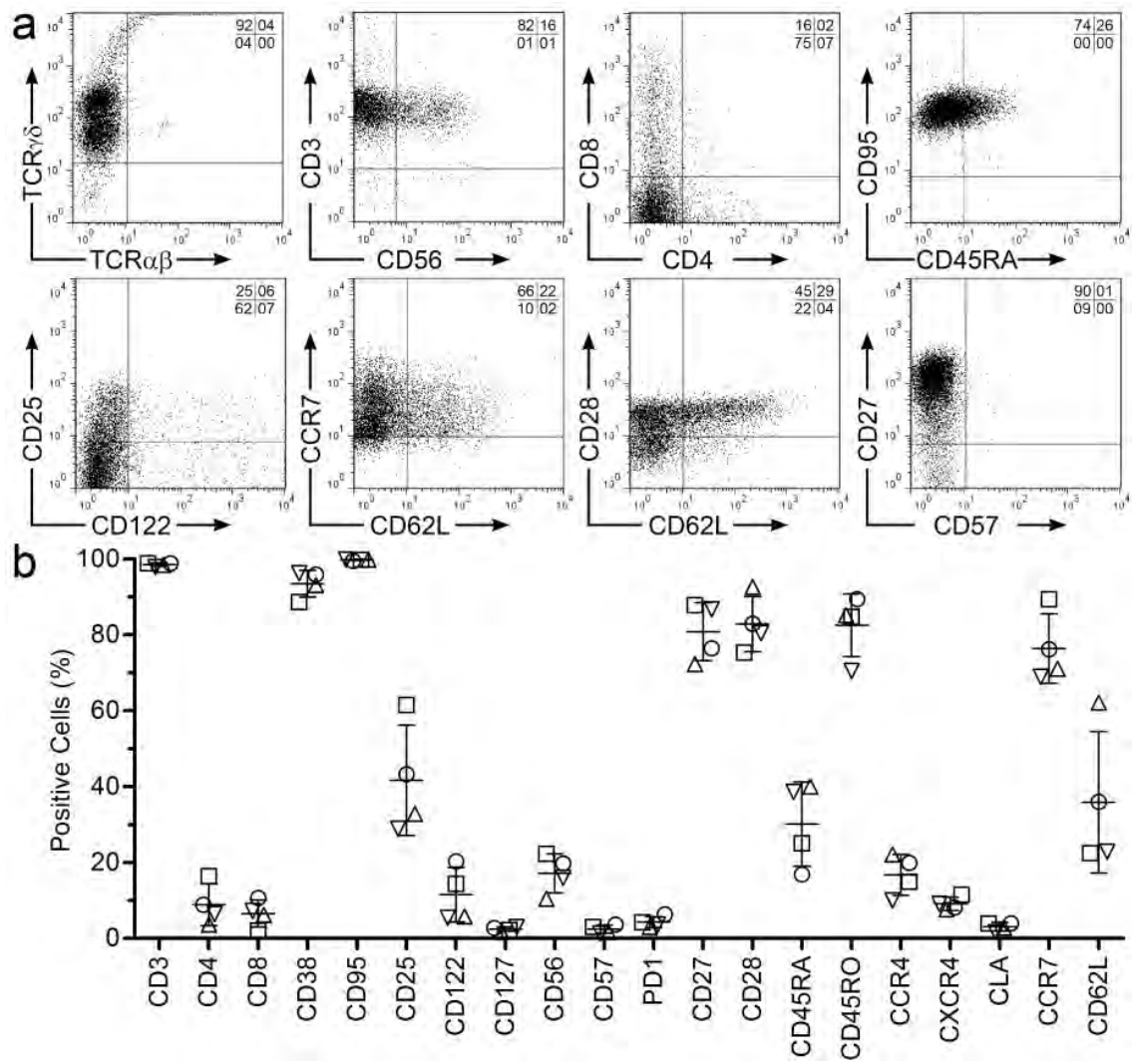
**Figure 33. Immunophenotype of Polyclonal γδ T cells Propagated on aAPC, IL2, and IL21.** (**a**) Gating (one representative of four donors is shown) and (**b**) frequency of T cell surface makers by flow cytometry of T cells at Day 22 of culture. Lines show mean (horizontal) ± SD (vertical) and symbols represent individual donors.

*IV.C.5.b. Immunophenotype of Vδ1, Vδ2, and Vδ3 Subsets*

It is of interest to identify differences amongst Vδ1, Vδ2, and Vδ3 lineages that could enable us to predict functional responses and therapeutic efficacy. In particular, distinct differences were observed in TCRγδ cell surface density and memory-associated markers. TCRγδ often stained as a two populations with distinct MFI when co-stained with CD3 (**Figure 27b**). Separation of Vδ1, Vδ2, and Vδ3 subsets clearly identified Vδ2 as the low (43 ± 9; mean ± SD; n = 4), Vδ3 as the medium (168 ± 40), and Vδ1 as the high (236 ± 56) MFI populations in TCRγδ staining (**Figure 34a**). CD4 and CD8 are not commonly expressed on γδ T cells, but there were differences detected in limited surface expression of both CD4 and CD8 between the separated subsets (**Figure 34b**). Vδ1 and Vδ3 cells consistently expressed more CD4 and CD8 than did Vδ2 cells (p = 0.001; Two-way ANOVA), and there were significantly more CD4$^+$ Vδ1 and CD8$^+$ Vδ3 cells than CD4$^+$ Vδ2 and CD8$^+$ Vδ2 cells, respectively (**Figure 34c and 34d**). CCR7 and CD62L mediate homing to the lymph nodes and other secondary lymphoid organs. CD8$^+$ T cells expressing CCR7 and/or CD62L were described as $T_{CM}$ cells but CCR7$^{neg}$CD62L$^{neg}$ were defined as $T_{EM}$ cells.(330, 331) Almost all Vδ1 and Vδ3 cells were CCR7$^+$CD62L$^{neg}$, but larger proportions of Vδ2 cells were CCR7$^{neg}$CD62L$^{neg}$ with roughly equal remaining proportions staining as single or double positive for CCR7 and CD62L, suggesting Vδ1 and Vδ3 were $T_{CM}$ and Vδ2 cells were mostly $T_{EM}$ (**Figure 34e**). CD27 and CD28 are both memory markers for CD8$^+$ T cells, especially in the absence of CD45RA, and have important roles as co-stimulatory molecules for T cell activation.(332) CD27 expression followed the order of Vδ1>Vδ3>Vδ2 but all were

>80% CD27$^+$ (**Figure 34f y-axes**). In contrast, there was almost no difference between the three Vδ populations in CD28 expression (**Figure 34f x-axes**). Human γδ T cell memory has been most extensively reported as combinations of CD27 and CD45RA expression where CD27$^+$CD45RA$^+$, CD27$^+$CD45RA$^{neg}$, CD27$^{neg}$CD45RA$^{neg}$, and CD27$^{neg}$CD45RA$^+$ correspond to T$_N$, T$_{CM}$, T$_{EM}$, and T$_{EMRA}$, respectively (**Figure 34g**).(151, 333) Indeed, these were the markers that showed the most convincing differences between the Vδ populations although all subsets contained at least some of each population. More specifically, the most T$_N$ cells were Vδ1, the most T$_{CM}$ were Vδ3, the most T$_{EM}$ cells were Vδ2, and virtually no T$_{EMRA}$ were detected (**Figure 34h**). Given these differences in surface memory phenotype, different functional abilities were expected from the γδ T cell subsets and a polyclonal approach to adoptive T cell therapy could utilize these different attributes as needed.
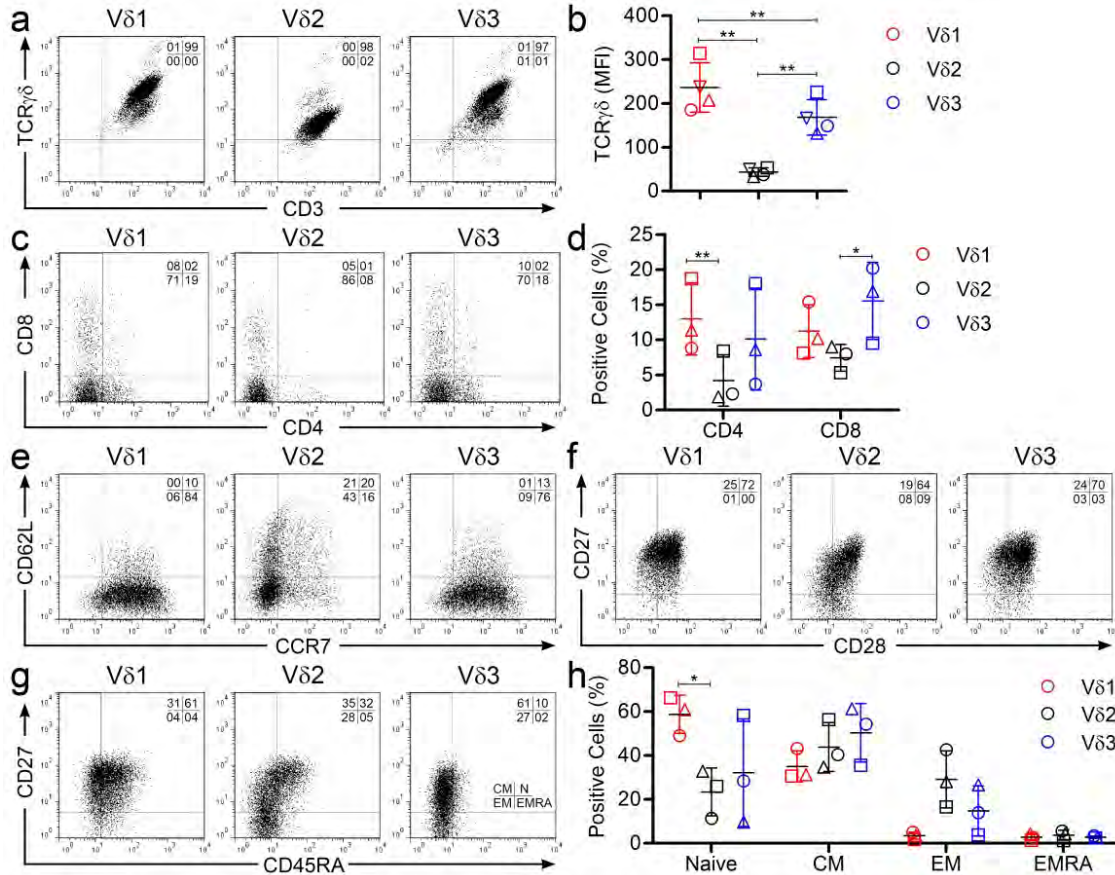
**Figure 34. Immunophenotype of Vδ Lineages Propagated on aAPC, IL2, and IL21.** After 15 days of proliferation as separated populations, Vδ1, Vδ2, and Vδ3 subsets were stained for lymphocyte markers. (**a**) Representative flow cytometry plots of CD3 (x-axes) and TCRγδ (y-axes) expression in Vδ1, Vδ2, and Vδ3 subsets (from left to right). (**b**) Mean fluorescence intensities (MFI) of TCRγδ staining in Vδ1 (red), Vδ2 (black), and Vδ3 (blue) subsets where each shape represents a different donor and data are mean ± SD (n = 4). (**c**) Representative flow cytometry plots of CD4 (x-axes) and CD8 (y-axes) expression in Vδ1, Vδ2, and Vδ3 subsets (from left to right) and (**d**) summary of frequencies in Vδ1 (red), Vδ2 (black), and Vδ3 (blue) cells where data are mean ± SD (n = 3) and each shape represents a different donor. Representative flow cytometry plots of (**e**) CCR7 (x-axes) and CD62L (y-axes), (**f**) CD28 (x-axes) and CD27 (y-axes), and (**g**) CD45RA (x-axes) and CD27 (y-axes) expression in Vδ1, Vδ2, and Vδ3 subsets (from left to right). Plots are representative of three normal donors. (**h**) Memory phenotypes based on CD27 and CD45RA displayed in lower right corner of Vδ3 in (**g**) where each shape represents a different donor and data are mean ± SD (n = 3).

138

IV.C.6. Polyclonal γδ T cells Secrete Pro-inflammatory Cytokines and Chemokines

To determine whether γδ T cells would foster an inflammatory environment during therapy, a multiplex analysis (27-Plex Luminex) of cytokines and chemokines was performed on polyclonal γδ T cells following culture on aAPC. LAC and mock activation was used as described in **Chapters II and III**. There was no significant production of anti-inflammatory $T_H2$ cytokines IL4, IL5, and IL13, and there was a small increase in IL10 production from baseline (**Figure 35a**). In contrast, IL1Ra, IL6, and IL17 were significantly secreted by γδ T cells and have roles together for $T_H17$ inflammatory responses (**Figure 35b**). Moreover, pro-inflammatory $T_H1$ cytokines IL2, IL12 (p70), IFNγ, and TNFα were all significantly produced by γδ T cells when TCR was stimulated compared to mock stimulated controls (**Figure 35c**). High expression of chemokines CCL3 (macrophage inflammatory protein-1α; MIP1α), CCL4 (MIP1β), and CCL5 (regulated and normal T cell expressed and secreted; RANTES) were also detected (**Figure 35d**). CCR5 binds to all three of these chemokines,(334) but only 6% ± 2% (mean ± SD; n = 4) of γδ T cells expressed this receptor. Nonetheless, recruitment of other immune cells expressing CCR5 is possible based on γδ T cell's production of CCL3, CCL4, and CCL5. In aggregate, TCR stimulation in γδ T cells led to a largely pro-inflammatory response desired for cell-based cancer therapies.
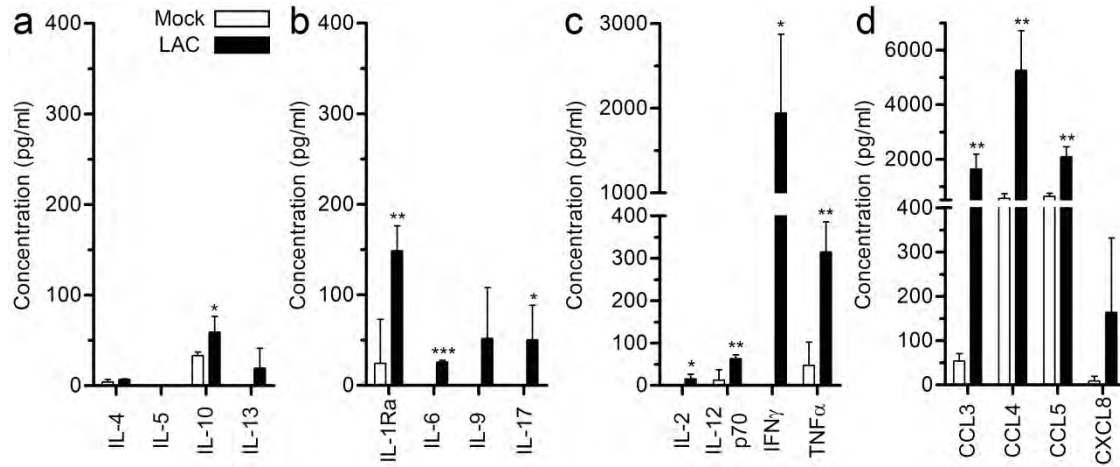
**Figure 35. Cytokines and Chemokines Secreted by Polyclonal γδ T cells.** At Day 22 of culture on aAPC/IL2/21, T cells were incubated with complete media (mock) or leukocyte activation cocktail (LAC; PMA/Ionomycin) for 6 hours at 37°C. Conditioned media was interrogated on 27-Plex Luminex array to detect cytokines and chemokines. (**a**) $T_H2$ cytokines, (**b**) $T_H17$ cytokines, (**c**) $T_H1$ cytokines, and (**d**) Chemokines. Data are mean ± SD from 4 healthy donors. Student's t-test performed for statistical analysis between mock and LAC groups for each molecule. *p<0.05, **p<0.01, and ***p<0.001

IV.C.7 TCRγδ Involvement in Vδ1, Vδ2, and Vδ3 Production of IFNγ

After establishing that polyclonal γδ T cells produced pro-inflammatory cytokines upon non-specific TCR stimulation, it was of interest to evaluate whether they would respond to tumor cells through their TCRγδ. IFNγ was produced most highly of all the cytokines interrogated by Luminex (**Figure 35c**), so it was chosen as a marker for γδ T cell response to OvCa in a classical intracellular cytokine staining (ICS) assay. Co-cultures with polyclonal γδ T cells and two different OvCa cell lines were incubated at 37$^{o}$C for 6 hours in the presence of the secretory pathway inhibitor Brefeldin-A (GolgiPlug) in order to trap IFNγ within the T cells. Parallel co-cultures were set up with (i) normal mouse serum (NMS) for negative control or (ii) neutralizing TCRγδ antibody (clone IMMU510) for 1 hour prior to co-culture and during the duration of co-culture. Surfaces of T cells were stained for CD3, TCRδ1, and TCRδ2 in order to separate Vδ1, Vδ2, and Vδ3 populations from tumor cells (**Figure 36a, 36b, and 36c**). Tumor cells alone and T cells without tumor cells served as negative staining controls. As anticipated, each Vδ subset produced IFNγ in response to OvCa in the NMS (negative blocking control) treated cells (**Figure 36d**). Furthermore, the amount of IFNγ produced followed the order Vδ2>Vδ1>Vδ3 as evidenced by IFNγ MFI of 855 ± 475, 242 ± 178, and 194 ± 182 (mean ± SD; n = 4), respectively. Addition of antibody neutralizing TCRγδ significantly inhibited IFNγ production by all three Vδ subsets where Vδ2 was most affected (**Figure 36d, 36e, and 36f**). Therefore, polyclonal γδ T cells responded to tumor cells indicating that they have specific anti-tumor effects through their TCRγδ.
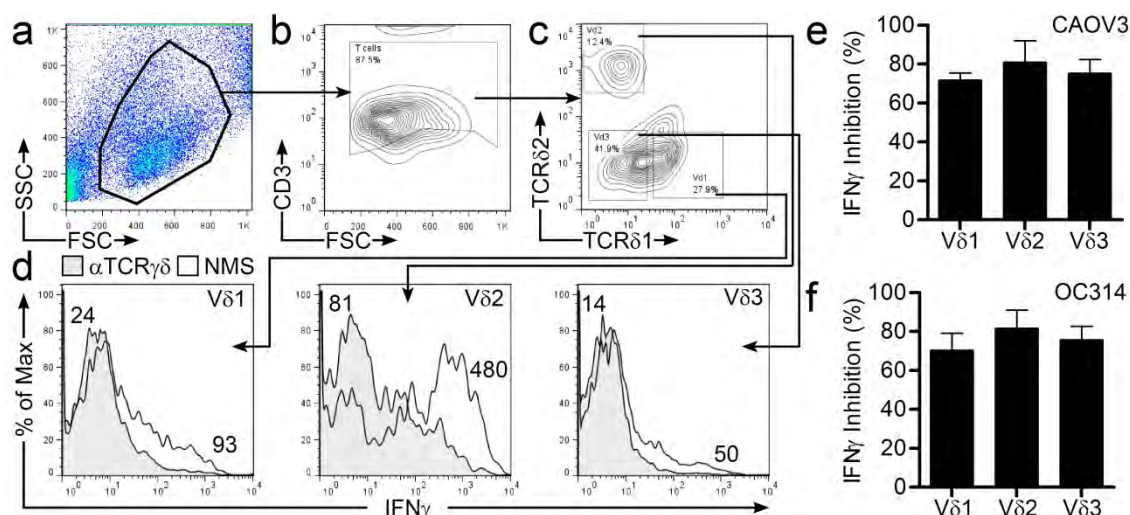
**Figure 36. TCRγδ-specific IFNγ Production by Vδ1, Vδ2, and Vδ3 Subsets.** Polyclonal γδ T cells were incubated for 1 hour prior to co-culture and during co-cultures with normal mouse serum (NMS; negative control) or neutralizing TCRγδ antibody (αTCRγδ; clone IMMU510). Co-cultures were initiated in the presence of the secretory inhibitor BrefeldinA (GolgiPlug) where polyclonal γδ T cells and one of two OvCa cell lines (CAOV3 or OC314) and were incubated at 37°C for 6 hours. Cells were stained for TCRδ1, TCRδ2, CD3, and IFNγ in order to gate each T cell subset and assess IFNγ production. The gating strategy was (**a**) separation of forward and side scatter (FSC and SSC, respectively) in activated T cell gate, (**b**) isolation of CD3$^+$ T cells from contaminating tumor cells in T cell gate, and (**c**) separation into Vδ1, Vδ2, and Vδ3 based on TCRδ1$^+$TCRδ2$^{neg}$, TCRδ1$^{neg}$TCRδ2$^+$, and TCRδ1$^{neg}$TCRδ2$^{neg}$, respectively. (**d**) Histogram comparisons of Vδ1, Vδ2, and Vδ3 gates (from left to right) co-cultured with CAOV3 and treated with NMS (open) or αTCRγδ (shaded). Numbers next to histograms are MFI. Flow plots are representative of 1 of 3 normal donors and of co-cultures with OC314 cells. Percent inhibition for each Vδ subset was calculated by the following equation: Inhibition (%) = 100 – 100 x [(MFI$_{TUMOR + T CELL}$ – MFI$_{T CELL ONLY}$)$_{αTCRγδ}$ / (MFI$_{TUMOR + T CELL}$ – MFI$_{T CELL ONLY}$)$_{NMS}$]. Data are mean ± SD (n = 3).

IV.C.8. Broad Anti-tumor Cytolysis by Polyclonal γδ T cells

After establishing that γδ T cells were functional in producing pro-inflammatory molecules, their ability to lyse a broad range of tumor cell lines was investigated against healthy donor cells and established hematological and solid tumor cell lines.

*IV.C.8.a. Polyclonal γδ T cells Lyse Hematological Tumors*

We previously established that γδ T cells could lyse B-ALL cell lines (Daudi-β2M, Kasumi2, and REH) but not healthy autologous or allogeneic B cells.(311) This observation was confirmed again with healthy autologous and allogeneic B cells, which were not lysed by polyclonal γδ T cells (**Figure 37a**). However, the same effectors were able to kill allogeneic B-ALL cell lines cALL2 and RCH-ACV (**Figure 37b**). T-ALL cell lines (Kasumi3 and Jurkat) were also sensitive to γδ T cell killing suggesting that γδ T cells could be used to kill T cell malignancies (**Figure 37c**). CML cell line K562 was also killed by γδ T cells and has been a well-known target for γδ T cell cytolysis.(303) Moreover, K562-derived clone#4 aAPC were lysed by γδ T cells, as expected (**Figure 37d**). Thus, polyclonal γδ T cells propagated on aAPC have anti-tumor immunity towards hematological malignances.
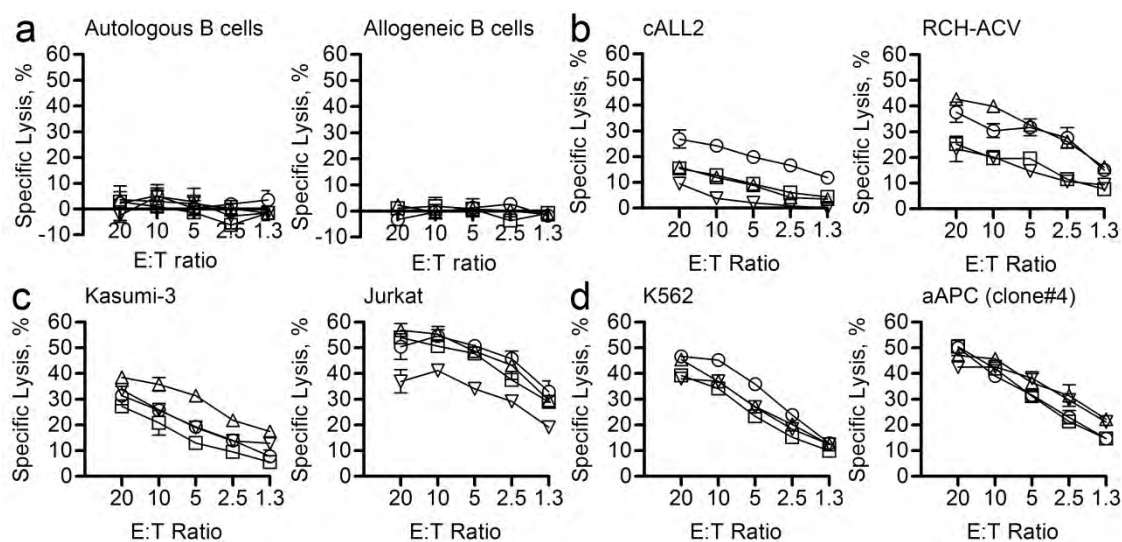
**Figure 37. *In vitro* Cytolysis of Hematological Tumor Cells by γδ T cells.** Standard 4-hour CRA were performed with increasing effector (γδ T cells) to target (E:T) ratios against (**a**) B cells from autologous donors or from an allogeneic donor (one of four representative donors), (**b**) B-ALL cell lines cALL2 and RCH-ACV, (**c**) T-ALL cell lines Kasumi3 and Jurkat, and (**d**) CML cell line K562 and its derivative clone#4 aAPC. Each line represents an individual effector where data are mean ± SD (n = 3 wells per assay).

*IV.C.8.b. Polyclonal γδ T cells Lyse Solid Tumors*

After establishing that polyclonal γδ T cells could lyse hematological tumor cells, solid tumor cell lines were evaluated for killing using standard 4-hour CRA. Established PaCa and OvCa cell lines were tested because of their high likelihood for sensitivity to anti-tumor immunity with a lack of current cellular therapies. Several PaCa cell lines (CaPan2, MiaPaCa2, Su8686, and BxPc3) cell lines were lysed by γδ T cells in a dose-dependent manner where BxPc3 cells were killed most efficiently (**Figure 38a**). Next, eight OvCa cell lines were lysed by polyclonal γδ T cells in the following order: CAOV3 > EFO21 > UPN251 > IGROV1 > OC314 > Hey > A2780 > OVCAR3 (**Figure 38b**). Moreover, there was an average of >60% maximum cytolysis observed against CAOV3 in one donor after 4 hours at an effector to target (E:T) ratio of 20:1. Therefore, polyclonal γδ T cells were able to kill solid tumors *in vitro* and other solid tumor cell lines may also be sensitive to cytolysis by γδ T cells.
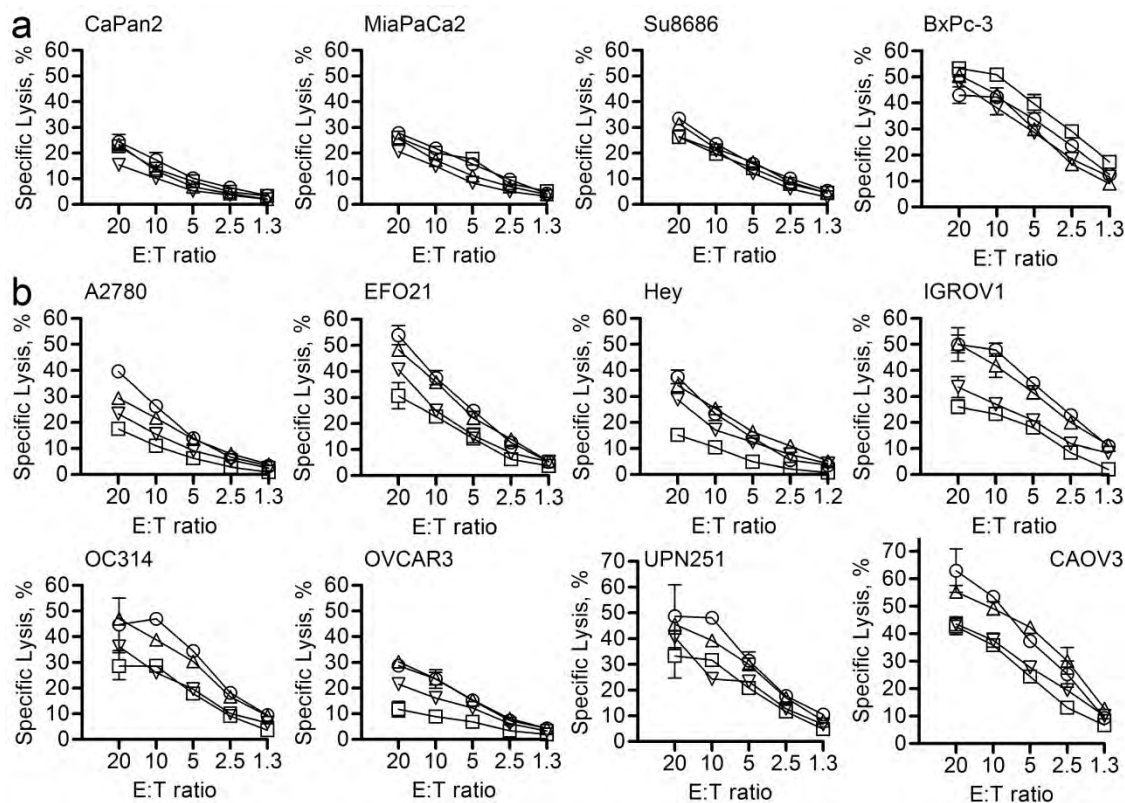
**Figure 38. *In vitro* Cytolysis of Solid Tumor Cells by γδ T cells.** Standard 4-hour CRA were performed with increasing effector (γδ T cells) to target (E:T) ratios against (**a**) PaCa cell lines CaPan2, MiaPaCa2, Su8686, and BxPc3 and (**b**) OvCa cell lines A2780, EFO21, Hey, IGROV1, OC314, OVCAR3, UPN251, and CAOV3. Each line represents an individual effector where data are mean ± SD (n = 3 wells per assay).

*IV.C.8.c. Mechanism of Tumor Cytolysis by γδ T cells was Multi-factorial*

We sought to determine if polyclonal γδ T cell cytolysis was directly dependent upon the TCRγδ by neutralizing killing with antibodies. Confounding these assays was the observation that γδ T cells displayed high levels of DNAM1 and NKG2D (data not shown), which can mediate cytolysis by both T cells and NK cells.(335, 336) Moreover, there was not a clear-cut choice for TCRγδ neutralizing antibody since the company information for TCRγδ-specific antibodies did not report on neutralization. In the end, the TCRγδ-specific antibody used for staining in this study (clone B1, BD Biosciences) and clone IMMU510 TCRγδ-specific antibody (IM) from Thermo Fisher were used for neutralization studies. Also, because there were many activating receptors (TCRγδ, DNAM1, NKG2D) on the γδ T cell surface, a pool of all antibodies was used for maximum inhibition and to assess if there was additivity or synergy between the receptors in killing. Hematological tumor cell line (Jurkat) and solid tumor cell line (OC314) were chosen as targets because of their reported expression of DNAM1 and NKG2D ligands and their sensitivity to cytolysis by polyclonal γδ T cells (**Figures 37c and 38b**).(337-339) An E:T ratio of 12:1 was chosen where effectors were pre-incubated with the antibodies and antibodies were present during the 4-hour CRA. NMS was used as a negative control and parallel wells were initiated without antibodies to determine maximum cytolysis for normalization purposes. Antibodies targeting NKG2D, DNAM1, and TCRγδ (clone B1) had minimal effect on reducing cytolysis against Jurkat (**Figure 39a**) and OC314 (**Figure 39b**) relative to NMS. However, there was a statistically significant increase in killing against Jurkat with DNAM1 antibody

and significant decrease in killing against OC314 with NKG2D antibody. In contrast, TCRγδ (IM) antibody significantly neutralized killing of both Jurkat and OC314 cells compared to NMS and reduced the killing by an average of 40% in both cell lines (**Figures 39a and 39c second bars from right**). Furthermore, a pool of all four antibodies (NKG2D, DNAM1, TCRγδ (B1), and TCRγδ (IM)) resulted in synergistic inhibition of γδ T cell cytolysis of Jurkat (65% ± 8%) and OC314 (71% ± 10%) cells (**Figures 39a and 39c bars to far right**). Moreover, dose-dependent inhibition was observed by both TCRγδ (IM) and pooled antibodies when concentrations were diluted from 3.0 μg/mL (shown in **Figures 39a and 39c**) to 1.0 μg/mL and 0.3 μg/mL against Jurkat (**Figure 39b**) and OC314 (**Figure 39d**). Similar results were seen with targeting IGROV1 (data not shown), which is also known to express DNAM1 and NKG2D ligands and was sensitive to polyclonal γδ T cell killing (**Figure 38b**).(337, 339, 340). In sum, these results suggested that killing by γδ T cells is multi-factorial with an emphasis on the TCRγδ to mediate cytolysis.
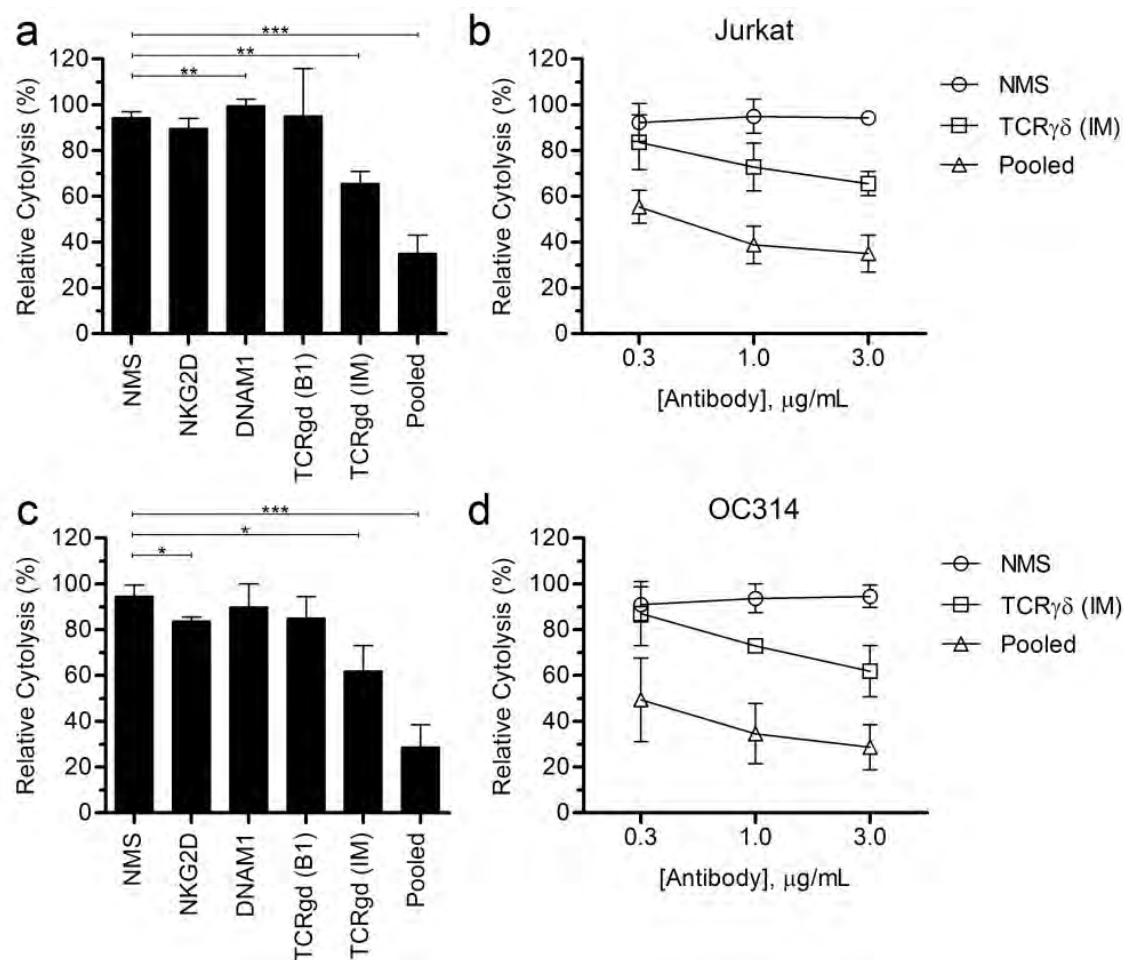
**Figure 39. Neutralization of Polyclonal γδ T cell Cytolysis.** Neutralizing antibodies to NKG2D, DNAM1, TCRγδ (B1), TCRγδ (IM) were used to block killing of Jurkat or OC314 tumor targets at an E:T ratio of 12:1 in standard 4-hour CRA. Antibodies were pre-incubated with T cells for 1 hour and kept in the CRA at 0.3, 1.0, or 3.0 μg/mL. NMS was used for antibody controls and specific lysis was normalized to wells without antibody to yield relative cytolysis as defined by: Relative cytolysis (%) = (Specific Lysis)$_{\text{With Antibody}}$ / (Specific Lysis)$_{\text{Without Antibody}}$ x 100. Relative cytolysis of Jurkat cells by (**a**) all antibodies at 3.0 μg/mL and (**b**) NMS, TCRγδ (IM), and pooled antibodies at tested concentrations. Relative cytolysis of OC314 cells by (**c**) all antibodies at 3.0 μg/mL and (**d**) NMS, TCRγδ (IM), and pooled antibodies at tested concentrations. Data are mean ± SD (n = 4). Two-way ANOVA with Bonferroni's post-tests were used for statistical analysis.

*IV.C.8.d. Importance for TCRδ in γδ T cell Cytolysis*

Because the separated Vδ subsets displayed differences in memory phenotype and cytokine production, it was of interest to evaluate their ability to directly lyse solid and hematological tumors. Acute killing was evaluated with standard 4-hour CRA against Daudi-β2M, Jurkat, K562, clone#4 aAPC, and OvCa cell lines (CAOV3, IGROV1, OC314, and UPN251) all of which displayed high levels of susceptibility to lysis by polyclonal γδ T cells (**Figures 37 and 38**). All eight tumor cell lines were lysed by the separated Vδ lineages, but a distinct order of lysis was observed against all targets where Vδ2>>Vδ3>Vδ1 in killing capabilities (**Figure 40**). As the phenotype indicated that Vδ1 cells were mainly naïve, it was expected that they would have the most limited cytolytic ability, which is what was observed. Likewise, $T_{CM}$ have less immediate effector function relative to $T_{EM}$ cells, and these memory populations were dominated by Vδ3 and Vδ1, respectively. Importantly, this was the first report of anti-tumor activity by Vδ3 cells. It was interesting that all three Vδ lineages lysed clone#4 aAPC roughly equally which supports their similar proliferation (**Figure 32**). Long-term killing assays were then set up to assess whether equivalent killing could be achieved during 48 hours of co-culture between Vδ subsets and OvCa cell lines CAOV3, OC314, and UPN251 (**Figure 41**). Indeed, >95% of CAOV3 and UPN251 cells were eliminated by all three subsets in two days. Likewise, 96% ± 4% of OC314 cells were killed by Vδ2 cells, and Vδ1 and Vδ3 achieved 76% ± 5% and 89% ± 5% (mean ± SD; n = 3) killing, respectively, in 48 hours of culture. Collectively, the Vδ subset lineage was

important for cytolysis in both acute and long-term conditions, and established that each

Vδ lineage propagated on aAPC was capable of tumor killing.
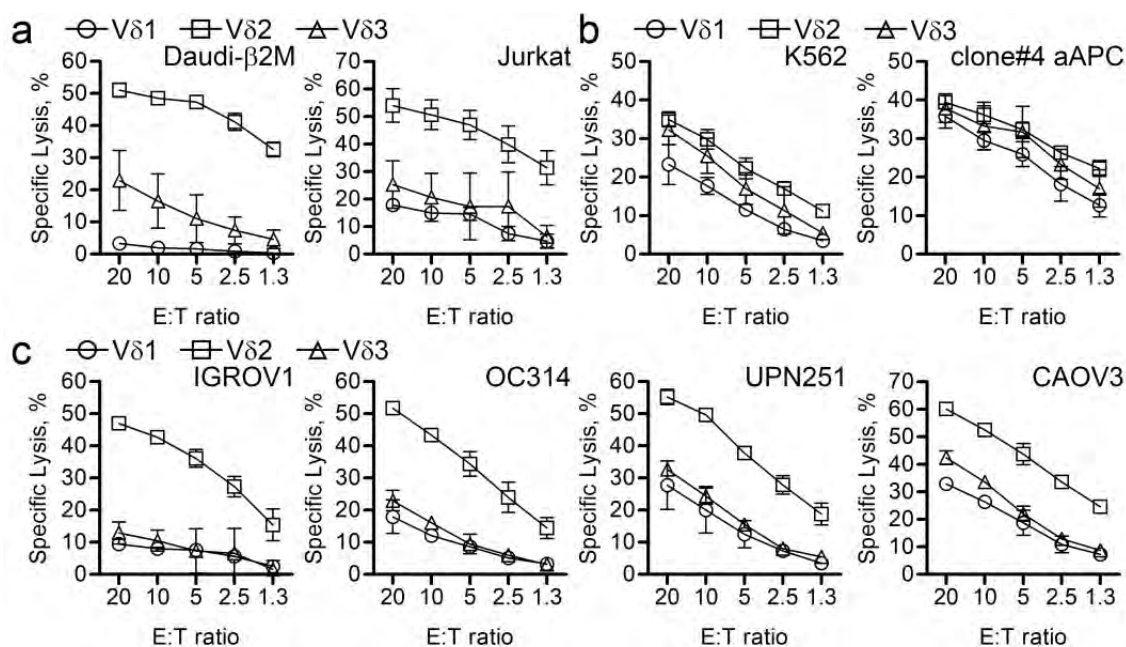
**Figure 40. γδ T cell Subset Acute Cytolysis.** Vδ subsets were tested in 4-hour CRA against (**a**) ALL cell lines Daudi-β2M and Jurkat, (**b**) CML cell line K562 and its derivative clone#4 aAPC, and (**c**) OvCa cell lines IGROV1, OC314, UPN251, and CAOV3. Vδ1 (circles), Vδ2 (squares), and Vδ3 (triangles) are displayed as mean ± SD from averaged triplicate measurements from four normal donors.
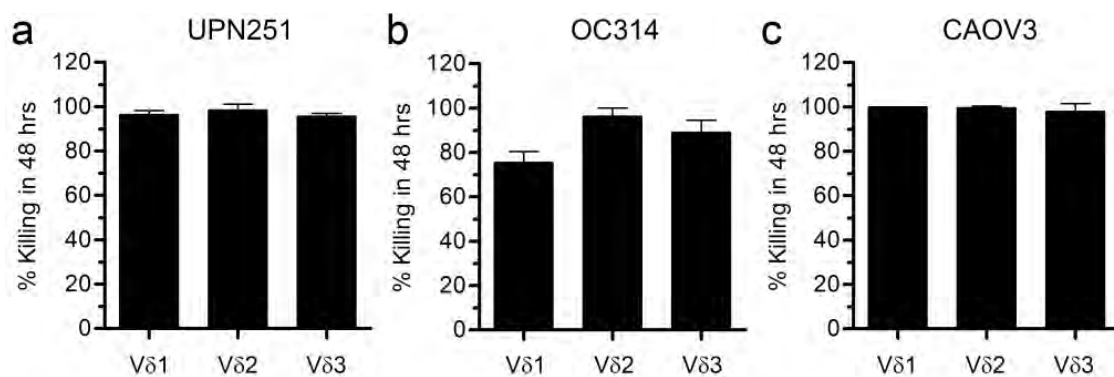
**Figure 41. γδ T cell Subset Long-term Killing.** CAOV3, OC314, and UPN251 cells were seeded in wells of 6-well plates and incubated overnight so that they would adhere to the wells. T cells from Vδ1, Vδ2, or Vδ3 subsets were then added and co-cultured in the wells with tumor cells for 2 days. Remaining adherent cells were enzymatically removed from the wells and counted for viable cells. Tumor cells without T cells were positive control and T cells without tumor cells was the negative control. Killing (%) = (Viable cells)$_{\text{Co-culture}}$ / (Viable cells)$_{\text{Tumor only}}$ x 100. Data are mean ± SD (n = 3).

IV.C.9. Clearance of Established Tumor Xenografts by Polyclonal γδ T cells

As polyclonal γδ T cells are being proposed as a therapy for cancer patients, a model to test their efficacy *in vivo* was evaluated. NSG mice were used for their ability to accept human tumor xenografts well and were injected with CAOV3-*ffLuc*-mKate tumor cells intraperitoneally (i.p.) then randomized into treatment groups to establish a model of high tumor burden. This was a model for advanced OvCa disease as many women with OvCa do not usually develop metastases outside of the peritoneal cavity but local tumor growth and ascites result in disease pathology.(37) After 8 days of engraftment (denoted Day 0) either PBS (negative control) or γδ T cells (escalating doses) were administered i.p. to the mice (**Figure 42**). Tumor burden was monitored during the experiment with non-invasive BLI following D-luciferin administration. Established tumors were clearly visible by BLI after 8 days of engraftment at Day 0 (**Figure 42a top panels**), which continued to grow in mock (PBS) treated mice (**Figure 42a bottom left panels**) but were eliminated in mice treated with polyclonal γδ T cells (**Figure 42a bottom right panels**) at 72 days post-treatment initiation. All mice treated with PBS had increased BLI flux measurements (p = 0.018) whereas polyclonal γδ T cell-treated mice had significantly decreased (p = 0.004) BLI flux (**Figure 42b**). Moreover, treatment with γδ T cells improved overall survival (p = 0.0001) compared to mock-treated mice where 90% of mice survived OvCa and hazard ratio for mice without treatment was 20.4 (**Figure 42c**). In sum, polyclonal γδ T cells were effective in treating cancer *in vivo* and represent an attractive approach to cell-based cancer treatment.
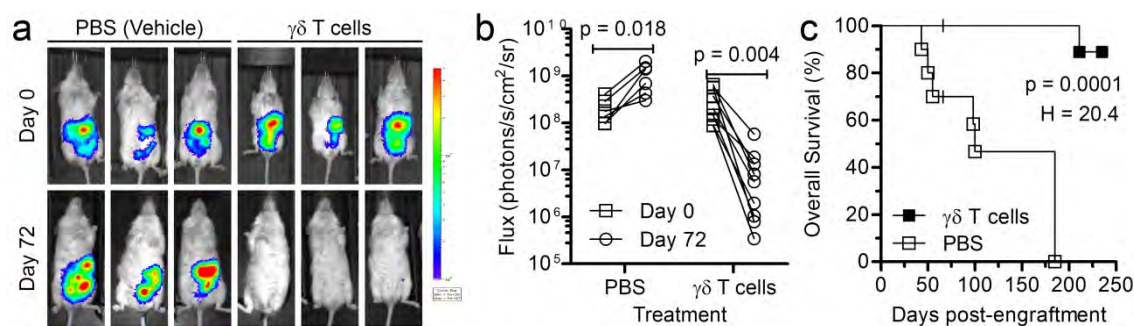
**Figure 42. *In vivo* Tumor Clearance by Polyclonal γδ T cells.** CAOV3-*ffLuc*-mKate tumor cells $(3x10^6)$ were injected i.p. into NSG mice at Day -8 and were allowed to engraft until Day 0 when treatment was started with either PBS (vehicle/mock) or polyclonal γδ T cells. Four doses were given with $3x10^6$, $6x10^6$, $10x10^6$, and $15x10^6$ on days 0, 7, 14, and 21, respectively, to create a dose escalation scheme. (**a**) BLI flux images at Day 0 (top panels) or Day 72 (bottom panels) in PBS-treated (left 3 panels) or polyclonal γδ T cell-treated (right 3 panels) mice. Mice displayed are representative of 10 total mice. (**b**) BLI flux measurements of mice at Day 0 (squares) and Day 72 (circles) where lines are drawn between the same mouse. Student's paired, 2-tailed t-tests were used for statistical analysis and p values are displayed above treatment groups. (**c**) Overall survival of mice treated with PBS (open squares) or polyclonal γδ T cells (closed squares). Gehan-Breslow-Wilcoxon Test was used to calculate p value. H = hazard ratio.

**IV.D. Discussion**

IV.D.1. Importance of Polyclonal γδ T cells for Immunotherapy

This study establishes clone#4 aAPC as a cellular platform for the sustained proliferation of populations of γδ T cells that exhibit broad reactivity against hematologic malignancies and solid tumors. T cells expressing certain Vδ TCR usage have been associated with clinical responses against cancer. For example, the Vδ1 TCR subset correlated with complete responses observed in patients with ALL and AML who underwent αβ T cell-depleted allogeneic HSCT.(302, 304, 305) However, Vδ1 cells have not been directly infused for therapy. This chapter established direct evidence that Vδ1 cells could mediate anti-tumor immunity and strengthens support for their use in adoptive T cell cancer treatments. In contrast to Vδ1 and Vδ3 cells, T cells expressing Vδ2 TCR have been directly infused and generated responses against solid and hematological tumors, but complete responses were unpredictable and sometimes not directly correlated to Vδ2 therapy (175, 318). Similarly, Vδ2 cells expanded in this chapter had the most immediate anti-tumor cytotoxicity and cytokine production, and aAPC-based expansions could build upon these early successes of Vδ2 T cell infusions. A role for T cells expressing Vδ3 TCR in targeting tumors is unknown, but these lymphocytes have been correlated with immunity to HIV and CMV.(165, 183) Thus infusion of this T-cell subset could be beneficial to immunocompromised patients. Importantly, these results are the first to directly show that Vδ3 cells have anti-tumor activity and this study could, therefore, represent a significant contribution to both translational research strategies and to immunologists studying γδ T cell function. In

aggregate, the data herein lend impetus to adoptive transfer of γδ T cells that maintain expression of all Vδ TCR types as investigational treatment for tumors and opportunistic viral infections.

IV.D.2. Potential Ligands for TCRγδ on aAPC

The molecules on aAPC that stimulate TCRγδ for their numeric expansion are not known. K562-derived aAPC express endogenous MICA and MICB molecules (329) which are ligands for both Vδ1 and NKG2D.(152) NKG2D was expressed (40% ± 16%; mean ± SD, n = 4) on aAPC-expanded γδ T cells that were also predominantly Vδ1 cells (**Figure 31**). Polyclonal γδ T cells also demonstrate expression (26% ± 7%) for other activating NK receptors (NKp30, NKp44, and NKp46), which may contribute to γδ T cell function. Two ligands described for Vδ2 TCR are surface mitochonrial $F_1$-ATPase and phosphoantigens, both of which are described in K562 cells.(171, 172, 297, 299) Indeed, enhanced responses of T cells expressing Vγ9Vδ2 were observed when K562 cells were treated with aminobisphosphonates,(172) and a similar strategy could be employed upon co-culture with clone #4 to increase the frequency of Vδ2 TCR usage.(173) Otherwise, patients receiving polyclonal γδ T cells could be primed to expand Vδ2 cells *in vivo* through administration of aminobisphosphonates. Now that aAPC have been established as a means to propagate polyclonal γδ T cells, these molecular questions can be answered and used for future therapies.

IV.D.3. Co-stimulation in Polyclonal γδ T cell Expansion

We introduced co-stimulatory molecules to improve the ability of aAPC to propagate γδ T cells. CD28 and CD137 (4-1BB) expressed on γδ T cells bind CD86 and CD137L, respectively, expressed on aAPC. The absence of both CD86 and CD137L abrogated γδ T-cell proliferation and expression of single co-stimulatory molecules only partially restored the ability of γδ T cells to proliferate (**Figure 28b**). The benefit of using other molecules' involvement in co-stimulation has not been evaluated to date. CD70 is expressed on γδ T cells (36% ± 15%) concurrently with its receptor CD27 (**Figure 33**), which may allow for *trans-* or *cis-* stimulation independent of the aAPC that does not express CD70. CD27 has been described as a marker for γδ T cells that produce IFNγ, and CD27$^{neg}$ γδ T cells commonly secrete IL17, a potent cytokine that has powerful, yet context-dependent anti-tumor activities.(127, 333, 341) Current studies are investigating whether other co-stimulation combinations, i.e. ICOS without CD86, can improve the propagation and/or change the phenotype of γδ T cells – especially in regards to improving production of IL17 that can have potent anti-tumor effects. It may be that a cocktail of cytokines and neutralizing antibodies is required to propagate IL17-producing γδ T cells, which was required for expansion of CD4$^+$ T$_H$17 cells *ex vivo* on stimulating beads.(326) Indeed, the addition of IL2 and IL21 was also crucial for the numeric expansion of γδ T cells so the strategy will likely need addition of these two exogenous cytokines (**Figure 28c**). In the end, the aAPC co-culture system provides a clinically relevant methodology to tailor the type of therapeutic γδ T cell produced for adoptive T cell therapy.

IV.D.4. Polyclonal γδ T cells Apparently Lack Allogeneic Responses to Healthy Tissue

An attractive therapeutic strategy is to employ third party allogeneic γδ T cells as an "off-the-shelf" therapy. This may be feasible, as γδ T cells have reduced potential to cause graft-versus-host disease (GvHD) resulting from inappropriate TCR-mediated recognition of normal host tissue (305). Unlike TCRαβ that recognizes peptides in the context of MHC, TCRγδ is not known to be subject to MHC restriction.(141, 298, 299) Thus, matching recipient and donor T cell MHC may not be needed, raising the possibility that propagated γδ T cells from one donor can be infused into multiple recipients. Autologous T cells expressing Vγ9Vδ2 TCR have been adoptively transferred and intravenous administration of aminobisphosphonates was used for *in vivo* numeric expansion of this T-cell subset.(175, 179, 318) To date, the infusion of allogeneic γδ T cell has not been reported. We have evaluated aAPC-expanded γδ T cells for allogeneic responses and are not able to detect such reactivity. For example, γδ T cells proliferate (**Figure 43a**) and secrete IFNγ (**Figure 43b**) when co-cultured with OKT3-loaded aAPC, but not when co-cultured with autologous or allogeneic B cells. Allogeneic tumor cell lines were lysed by our γδ T cells, but healthy B cell donors were spared (**Figures 24a and 37a**). Further, formation of colonies from hematopoietic stem cells was inhibited by allogeneic NK cells, but not by allogeneic γδ T cells (**Figure 43c**). Autologous EBV-transformed LCL stimulated γδ T cells suggesting they may react with EBV antigens (data not shown) as indicated by previous studies.(342, 343) Bi-specific αβ T cells expressing CARs specific for GD2 or CD19 and grown on LCL have shown excellent anti-tumor immunity and could be applicable for the γδ T cell

population using aAPC.(236, 344) The ability to infuse donor-derived γδ T cells when needed, rather than wait the availability of an autologous product raises the therapeutic potential of this T-cell subset. This adds to our development of "off-the-shelf" cells as we previously reported that zinc finger nucleases can be used to eliminate expression of TCRαβ to help generate "universal" CAR^{+} T cells.(345)
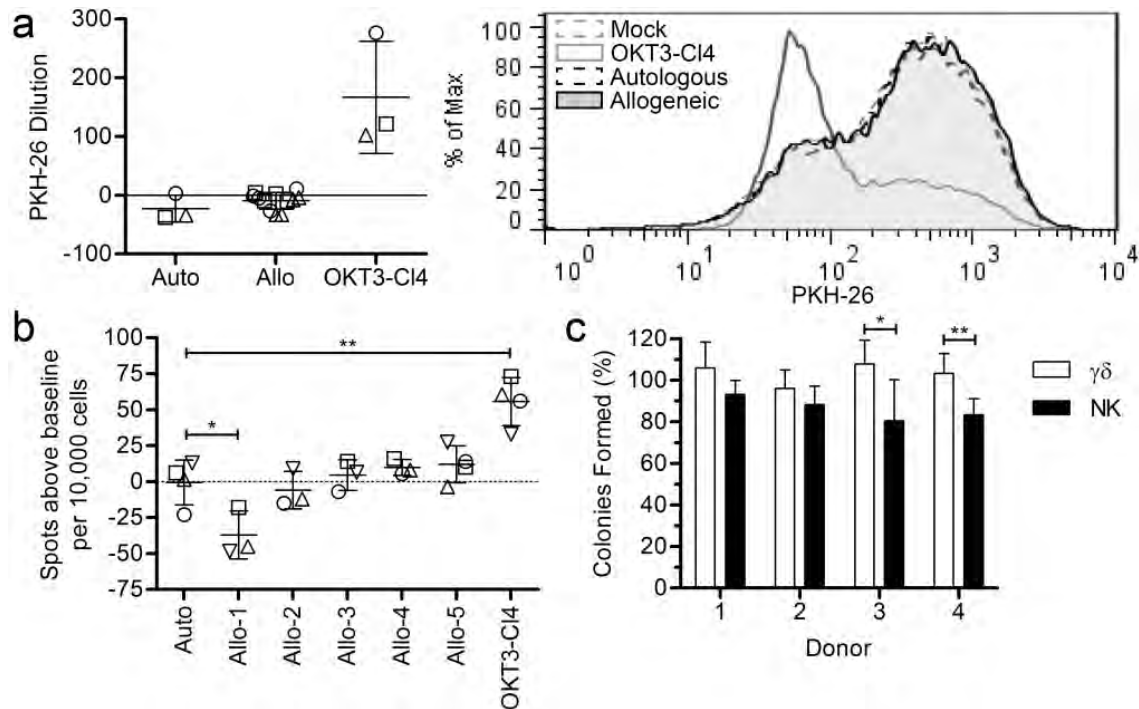
**Figure 43. Absence of Allogeneic Responses by Polyclonal γδ T cells to Partially Mis-matched Donors' Healthy Cells. (a)** Polyclonal γδ T cells were labeled with red fluorescent dye (PKH-26) and co-cultured with (i) media only (mock), (ii) autologous B cells, (iii) allogeneic B cells from normal donors (n = 5), or (iv) OKT3-loaded clone#4 aAPC (positive control) for 3 days at 37°C without exogenous cytokines. Proliferation was measured by dilution of PKH-26 dye MFI and each group was normalized to mock treated T cells after gating for CD3⁺TCRγδ⁺ cells. Each shape represents a polyclonal γδ T cell effector (n = 3). Representative flow cytometry plot is displayed to the right. **(b)** The same co-cultures set up in (**a**) were initiated overnight in an IFNγ ELISpot assay plate, except that cells were not labeled with PKH-26. Spots were enumerated and normalized to mock-treated cells for each donor, which is represented by an individual shape. **(c)** Hematopoietic stem cell (HSC) colony forming unit assays were set up with co-cultures of donor-matched NK cells or γδ T cells and PBMC containing a fixed number of HSC and co-cultures were added to semi-solid media supplemented with cytokines for colony formation. HSC cultures without co-cultured lymphocytes were used as negative controls for inhibition of colony formation and to normalize co-culture colony formation. Student's paired, 1-tailed t-tests were used for statistical analysis. *p<0.05 and **p<0.001.

IV.D.5. Application of Polyclonal γδ T cells for Immunotherapy

These data demonstrate that our aAPC can be used to generate large numbers of γδ T cells that maintain polyclonal TCR repertoire and have an ability to kill tumor cells. Clone#4 has been produced as a master cell bank and thus there is a clear path to generating clinical-grade γδ T cells for human application. A polyclonal approach to γδ T cell immunotherapy is supported by the ability to of aAPC generate $T_N$, $T_{CM}$, and $T_{EM}$ γδ T cells from Vδ1, Vδ2, and Vδ3 lineages (**Figure 34**) that could then produce a range of effector functions including production of pro-inflammatory cytokines (**Figures 35 and 36**), exerting direct cytotoxicity against tumors (**Figures 37, 38, 39, 40 and 41**), and eliminating solid tumor xenografts (**Figure 42**). Thus, immediate tumor cytotoxicity can be achieved mainly through effector and $T_{EM}$ cells and long-lived anti-tumor immunity could be repopulated in patients with $T_N$ and $T_{CM}$ γδ T cells. Clinical trials can now, for the first time, test the efficacy of polyclonal γδ T cell transfers in cancer treatments of both solid and hematological tumors.

# CHAPTER V

## General Discussion and Future Directions

### V.A. Dissertation Summary

The central aim of this dissertation was to develop and test novel cellular immunotherapies for cancer treatment. This was tested in three independent specific aims. First, ROR1-specific CARs were able to re-direct $\alpha\beta$ T cells towards leukemia without affecting normal B cells, and this represented an improvement from current CD19-specific CAR strategies that result in normal B cell aplasia (**Chapter II**). Current CD19-specific CAR and CD19$^+$ aAPC are currently in clinical trials at MD Anderson and were the fastest way to translate a strategy to use CAR$^+$ $\gamma\delta$ T cells for immunotherapy. Therefore, the second approach used polyclonal $\gamma\delta$ T cells expressing TCR$\gamma\delta$ with anti-tumor reactivity as sentinels of CD19-specific CAR anti-tumor immunity. These CAR$^+$ $\gamma\delta$ T cells may have clinical bi-specific anti-leukemia efficacy due to targeting the tumor through both TCR and CAR (**Chapter III**). The last aim evaluated the broad anti-tumor activity of polyclonal $\gamma\delta$ T cells expanded on aAPC, and established that they can be an effective option for leukemia, PaCa, and OvCa (**Chapter IV**). The translation of these pre-clinical methods into the clinical trials will give people facing cancer treatment new, safe, and effective options.

## V.B. Combinational Cellular Immunotherapies

Using more than one cell immunotherapy product in therapy may lead to therapeutic additivity, or better yet, synergy. Indeed, clinical trials have already combined HSCT with CD19-specific CARs to target B-cell leukemia.(263, 346) The trials are still in the enrolling stages, so it will take time to determine whether they are better than historical controls. Similar to HSCT and CD19-specific CAR^{+} T cells, CARs can be paired to other cellular products to increase anti-tumor efficacy. For instance, polyclonal γδ T cells had inherent anti-tumor immunity towards ovarian and pancreatic cancers (**Chapter V**) and ROR1 is a TAA expressed on both PaCa and OvCa where ROR1^{+} OvCa cells were lysed by ROR1-specific CAR^{+} T cells (**Figure 16c**),(67) so a combinational immunotherapy of ROR1-specific αβ T cells and polyclonal γδ T cells could be used. In fact, the 4A5 mAb specific for ROR1 and from which the CAR was derived detected ROR1 at some level in 11 of 12 OvCa cell lines (**Figure 6c and data not shown**). Given the potent anti-tumor activity of polyclonal γδ T cells towards OvCa (**Figure 38b**), the two approaches could be done together to increase tumor clearance. Moreover, patients with low ROR1 antigen expression and resistance to γδ T cell-mediated cytolysis may be sensitive to synergistic killing by ROR1-specific CAR[+] αβ T cells and polyclonal γδ T cells. Also, γδ T cells are unlikely to participate in GvHD in allogeneic transplantation, so a universal bank of polyclonal γδ T cells could be established that was known to have high anti-tumor immunity or containing a particular set frequency of Vδ1, Vδ2, and Vδ3 populations with maximum efficacy.(305) Polyclonal γδ T cells could also be used as front-line therapy before addition of HSCT, CAR^{+} T cells, TILs, etc. in order to prime the tumor microenvironment for adaptive

immune cells with broader tumor specificity or to reveal neo-tumor antigens. Furthermore, the bystander effects of γδ T cells in the microenvironment are largely unknown, and tumor lysis could lead to other resident cell types, e.g. NK cells, macrophages, DCs, etc. to have renewed reactivity to the tumor.(347) Indeed, B-ALL cell lines coated with mAb were lysed by CD16^{+} Vγ9Vδ2 cells via antibody-dependent cell-mediated cytotoxicity (ADCC), and subsequently the Vγ9Vδ2 had APC function to generate antigen-specific CD8^{+} αβ T cell responses to known B-ALL peptides, e.g. PAX5.(348, 349) The advantage of polyclonal γδ T cells expanded on aAPC is that there may be sufficient direct tumor lysis that ADCC would not be necessary. However, the APC function of aAPC-expanded polyclonal γδ T cells has not yet been studied. Lastly, melanoma may be an ideal target for combinational cellular immunotherapy because it is one of the most responsive tumors to immunotherapy and many T cells specific to melanoma peptides, e.g. MART1 and gp100, have been well characterized for rapid detection of antigen-specific responses. As aAPC have already been adapted for melanoma TIL studies (Forget MA, unpublished observation),(274) it is a logical next step to evaluate whether polyclonal γδ T cells can induce antigen-specific CD8^{+} T cell responses to melanoma. If successful, this approach could impact the TIL expansion protocols to adapt them to a wider range of patients. In aggregate, there are many combinatory approaches that can be taken to increase the therapeutic payload to cellular immunotherapy.

## V.C. Generation of IL17-producing T cells for Adoptive Immunotherapy

IL17 has been shown to have potent anti-tumor effects when used in the tumor microenvironment, and therefore secretion of IL17 by transferred T cells homing to the tumor may have potent anti-tumor immunity.(321, 323, 350) T cells that produce IL17 can be mutually exclusive from those who produce IFNγ. Indeed, most of the T cells expanded on aAPC in this dissertation, with or without CARs, produced IFNγ, and the expanded γδ T cells secreted IL17 in diminished quantities compared to IFNγ (**Figures 23 and 35**). CD27 has been a marker for these cytokines in γδ T cells where CD27^{neg} and CD27^{+} are associated with IL17 and IFNγ, respectively.(333, 351, 352) It holds then that ~80% of polyclonal γδ T cells stain positive for CD27 (**Figures 33 and 34**). CD28 co-stimulation was shown to inhibit $T_H17$ polarization in CD4^{+} T cells through ICOS co-stimulation,(326) and so it may be that CD86 co-stimulation by aAPC and/or CD28 endodomains in the CAR lead to polarization towards IFNγ in polyclonal γδ T cells, CAR[+] γδ T cells, and CAR[+] αβ T cells. Replacement of CD28 for ICOS in the CAR(s) and CD86 for ICOSL in the aAPC can be tested to see if these can generate T cells that secrete IL17. Another strategy comes out of the observation that ROR1-specific CAR^{+} T cells signaling through CD137 produce less IFNγ than do those signaling through CD28 (**Figure 15**). This may be due to (i) CD137 signaling yielding less inflammatory cells or (ii) CAR-CD137 cells expressed other cytokines that have yet to be detected. Clinical trials out of The University of Pennsylvania (PI: June, CH) using CD19-specific CAR^{+} T cells for ALL and CLL treatment have shown that responders had high serum IL6.(4, 7) This cytokine has importance for macrophages, inflammatory response (of particular interest in his trials as patients underwent massive

166

fevers from T cells attacking their leukemia), and polarization of $CD4^+$ T cells from $T_{REG}$ to $T_H17$.(127) In regards to the latter, release of immunosuppression by $T_{REG}$ and production of IL17 could explain these impressive complete responses. **Chapter II** did not directly evaluate the influence of $T_{REG}$ cells on $CAR^+$ T cell function or IL6 and IL17 production, but experiments using intracellular cytokine staining or multiplex arrays can be used to pursue this line of questioning. Development of an aAPC-based expansion of IL17 secreting T cells would allow for direct testing of their benefit relative to IFNγ-producing cells, and may lead to rationales to use one or both of them in the clinic for cancer therapies.

## V.D. Importance of Polyclonal γδ T cells to Immunology

One of the major accomplishments of this dissertation was creating a method for expanding polyclonal γδ T cells (**Chapter IV**), which has broader applications outside of immunotherapy to the immunology and cancer biology fields. For example, few mAb exist that are specific for TCRγδ isotypes, which limits their detection in correlative studies and other assays.(165) Given the ability of aAPC to expand large numbers of polyclonal γδ T cells, mice can be immunized to generate mAb specific for desired TCRγδ isotypes, e.g. Vδ3 and Vγ isotypes outside of Vγ9. Commercial and academic use of these detection antibodies have tangible outcomes, including diagnostic and/or prognostic profiling of γδ T cell TIL within tumors. Other major unknowns are the ligands for many TCRγδ heterodimers. Generation of γδ T cell clones could be used to determine the specific ligands of Vδ/Vγ combinations and therefore lead to future

167

studies on γδ T cell affinity towards a particular disease. Moreover, the ligands on the K562-derived aAPC that TCRγδ binds are unknown. Likely candidates include IPP (Vδ2) and MICA/B (Vδ1) but their exact roles have not been determined.(155, 172) Elucidation of these interactions could assist attempts to tailor the aAPC for total γδ T cell expansion, expansion of a particular γδ T cell lineage, or polarization towards a certain γδ T cell phenotype. Thus, aAPC could be an excellent source for the study of fundamental γδ T cell immunobiology and could yield answers not currently accessible because of limited starting cell numbers and ineffective polyclonal expansion protocols.

## V.E. Potential Benefits and Issues with Cellular Immunotherapy

Although promising, there may be some limitations to the immunotherapies created in this dissertation. First, patients with advanced B-cell leukemia disease often have few T cells in their peripheral blood, and have even fewer γδ T cells.(6) In some cases, the residual autologous T cells are functionally unresponsive and difficult to expand to clinically-relevant doses.(353) Preliminary studies using CD19-specific CAR have indicated that CAR$^+$ T cells can be generated from CLL patients with <5% T cells at the start of culture (Huls MH, unpublished observation). Other options would be to use haplo-identical or MHC-matched T cells. However, this is not always feasible, so allogeneic γδ T cells could be an ideal choice because of they are generally thought to recognize antigens outside of MHC-restriction.(304, 342) Of course, if normal hematopoiesis resumes in the patients then the γδ T cell graft may be rejected, but there may still be a therapeutic window. Another unknown is whether γδ T cells will be

subjected to the same regulation by $T_{REG}$ cells or other immunosuppressive forces. Some γδ T cells have been reported to have immunosuppressive function, and it would be of interest to identify these cells and eliminate them from the adoptive T cell product prior to infusion.(354) The tumor microenvironment is also of interest because it often contains hypoxic areas containing malignant cells resistant to conventional treatments.(355, 356) In preliminary experiments, the co-culture system was adapted to assess γδ T cell proliferation as a function of oxygen tension. No difference in proliferative capacity (p = 0.404) was observed when the cultures were in hypoxia (1% $O_2$) or normoxia (20% $O_2$) and stimulated with clone#4 aAPC, IL2, and IL21, indicating that γδ T cells have potential to operate within the bone marrow or hypoxic tumor milieu (**Figure 44**). Thus, administration of graded doses of autologous and allogeneic γδ T cells in humans will test the ability of γδ T cells to home and recycle effector function in the tumor microenvironment. In the end, clinical trials will be the ultimate test of whether these potential pitfalls out weight the anti-tumor benefits to cancer patients.
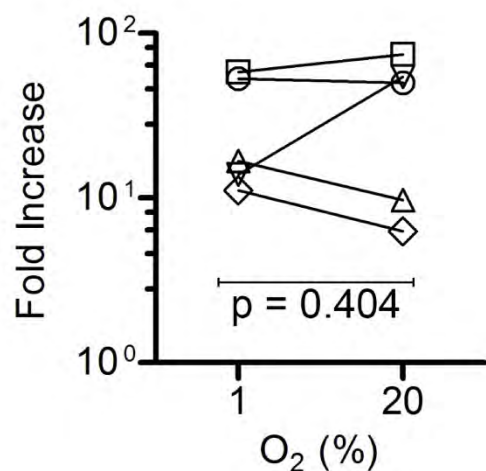
**Figure 44. Proliferation of γδ T cells in Hypoxia Compared to Normoxia.** Co-cultures were initiated in parallel with γδ T cells and aAPC in the presence of exogenous IL2 and IL21 in incubators set with either 1% $O_2$ (hypoxia) or 20% $O_2$ (normoxia) and were normalized to starting quantities 10 days after culture initiation. Student's paired, 2-tailed t-test was used for statistical analysis.

**V.F. Clinical Applications of Dissertation Immunotherapies**

As of June, 2013 there are immediate plans to use immunotherapies detailed in **Chapters II and IV** in the clinic. A Phase I clinical trial was written to co-administer autologous ROR1RCD28 and ROR1RCD137 T cell populations into CLL patients after lymphodepletive (Cytoxan and Fludarabine) chemotherapy. Proof-of-principle studies have established protocols for expanding CAR$^+$ T cells from patient samples by using an "electroporation-then-sort" strategy used for growing CAR$^+$ γδ T cells (**Chapter III**). Patient PBMC will be electroporated with SB transposase and SB transposase plasmids and sorted on paramagnetic beads the following day to deplete CD19$^+$ T cells. Co-culture on aAPC led to CAR$^+$ T cell growth in the translation research lab (TRL) built to translate lab protocols to the current good manufacturing practices (cGMP) facility. As more data has arrived, the support for utilizing only ROR1RCD137 in the clinical trial has gained momentum and may be the treatment modality tested instead of a competitive re-population experiment of both CAR$^+$ T cell populations. This investigational new drug (IND) application passed rigorous examination by the National Institutes of Health (NIH) Recombinant DNA Advisory Committee (RAC) with approval in December 2012. Review at the MD Anderson IRB is underway before sending the trial for final IND approval by the Food and Drug Administration (FDA). A second CAR trial has been proposed for treatment of leukemia with both ROR1-specific T cells and the chemotherapy dasatinib, which leads to increased surface expression of ROR1 in t(1;19) B-ALL cells and could minimize the risk for ROR1 antigen escape.(13) In regards to **Chapter IV** translation, a compassionate IND (CIND) has been written to treat a late stage CLL patient with autologous or allogeneic polyclonal

γδ T cells in the case that the autologous γδ T cells do not proliferate or respond to the tumor. If allogeneic γδ T cells are infused into this patient, this will represent the first time that purified polyclonal γδ T cells from an allogeneic host were ever infused into a human. There is great optimism that the polyclonal γδ T cells can home to secondary lymphoid tissues harboring CLL and that they can eliminate the leukemia. These two trials are, hopefully, the beginning of the trials to come that will apply ROR1-specific T cells, CAR$^+$ γδ T cells, and polyclonal γδ T cells for human cancer immunotherapies.

<u>**CHAPTER VI**</u>

**MATERIALS AND METHODS**

**VI.A. DNA Plasmids and Construct Cloning**

All plasmids in this study were propagated in dam$^{-/-}$ bacteria (C2925, Invitrogen, Grand Island, NY) and purified as single cell bacteria clones with EndoFree Plasmid Maxi Kit (Qiagen, Valencia, CA). Plasmids were cleared for transfection when (i) identity was confirmed by analytical digestion, (ii) samples were negative for endotoxin, and (iii) had spectrophotometer readings of $1.80 < A_{260}/A_{280} < 2.00$.

<u>VI.A.1. Tumor Antigens</u>

*VI.A.1.a. ROR1*

The extracellular and transmembrane domains of ROR1 (Accession: NM_005012), termed dROR1, were cloned into a SB vector (pSBSO). The open reading frame (ORF) was codon optimized for expression in human cells and cloned into a shuttle vector (pMK-RQ) by GeneArt (Invitrogen). Codon-optimized dROR1/pMK-RQ and GlySer-EGFP-mIgG1(CooP)/pSBSO plasmids were digested with *NheI* and *XhoI* restriction enzymes and were purified from pMK-RQ and GlySer-EGFP-mIgG1 fragments, respectively, by gel electrophoresis. Purified dROR1 and pSBSO fragments were ligated with T4 DNA Ligase (Promega, Madison, WI) to create dROR1/pSBSO plasmid, which was then amplified in the presence of kanamycin for large-scale

purification. Identity of the purified plasmid was confirmed with digestions of (i) *ClaI*, (ii) *ClaI* and *SmaI*, (iii) *PvuII*, and (iv) *PvuI* and *SmaI* enzymes to distinguish between parental plasmids and dROR1/pSBSO.

*VI.A.1.b. CD19*

The extracellular and transmembrane domains of human CD19 (Accession: M84371), termed Delta-CD19, were cloned into a pSBSO with linked F2A cleavage site and neomycin resistance (NeoR) for enforced dCD19 expression (performed by Olivares S). As with dROR1, the ORF was codon optimized for expression in human cells and cloned into a shuttle vector by GeneArt. In order to create the final vector, codon-optimized dCD19 from plasmid vector Delta-CD19(CoOp)-F2A-SStomato/pSBSO and Neomycin resistance from plasmid vector Myc-FFLuc(CoOp)-Neo/pSBSO, were digested with *ZraI*/*SpeI* and *EcoRV*/*SpeI* restriction enzymes respectively. The fragments (Neo-insert and Delta-CD19(CoOp)-F2A-X/pSBSO-vector) were purified by gel electrophoresis. Purified fragments were ligated with T4 DNA Ligase to create Delta-CD19(CoOp)-F2A-Neo/pSBSO plasmid, which was then amplified in the presence of kanamycin for large-scale purification. Identity of the purified plasmid was confirmed with digestions of *SacI* restriction enzyme to distinguish between parental plasmids and Delta-CD19-F2A-NeoR/pSBSO.

VI.A.2. Co-stimulatory Molecules

*VI.A.2.a. CD86 and CD137L*

The entire ORF for CD86 (Accession: EF064748.1) and CD137L (Accession: NM_003811.3) were codon optimized and synthesized by GeneArt and were then cloned into pSBSO (performed by Ang S).

*VI.A.2.b. IL15-IL15Rα Fusion Construct*

This construct will produce an IL15 that is membrane-bound, but also presented in the context of IL15Rα. A fusion of IL15 (NM_000585.4) to the full length IL15Rα (NM_002189.3) was constructed with a serine-glycine linker and a C-terminal Flag (x3) motif attached to generate membrane bound IL15 (mIL15). The signal peptides for IL15 and IL15Rα were omitted and the IgE signal peptide (gb|AAB59424.1) was used for the mIL15 fusion protein. As with dROR1, mIL15 was codon optimized and synthesized by GeneArt and was then subcloned into GlySer-EGFP-mIgG1(CooP)/pSBSO using *NheI* and *XhoI* restriction sites.

VI.A.3. Chimeric Antigen Receptors

Cloning of second generation CD19-specific CAR signaling through CD28 and CD3ζ (CD19RCD28) has been previously described.(57, 272, 281) The CAR was modified to replace CD28 endodomain for CD137 endodomain as a synthetic cDNA sequence

(GeneArt) that was cloned back into the original plasmid with *SmaI* and *SpeI* restriction endonucleases to create another second generation CD19-specific CAR signaling though CD137 and CD3ζ (CD19RCD137). These plasmids were further manipulated to contain "SIM" and "FRA" oligonucleotides at the 3' end of the CD19RCD28 and CD19RCD137 transposons, respectively, by shuttling the entire CARs into new pSBSO backbones with *NheI* and *XhoI* enzymes (CD19-specific CAR work performed by Olivares S). Heavy and light chain immunoglobulin sequences from the 4A5 mAb hybridoma were provided by Dr. Thomas J Kipps (UCSD) and were used to assemble the following ROR1R sequence *de novo* (GeneArt) from 5' to 3' (i) murine IgGκ signal peptide, (ii) $V_L$, (iii) Whitlow linker, (iv) $V_H$, and (v) the first 73 amino acids of the IgG4 stalk, and ROR1R sequence was shipped to MD Anderson as ROR1R(CoOp)/pMK-RQ plasmid. Amplification of ROR1R fragment from ROR1R(CoOp)/pMK-RQ was done by PCR with the following primers: ROR1RCoOpF (GCTAGCCGCCACCATGGGCTGGTCCTGCATC) and ROR1Rrev (GCTCCTCCC GGGGCTTTGTCTTGGC). The PCR product was cloned into pCR4-TOPO with TOPO TA Cloning Kit (Invitrogen) to generate ROR1R(CoOp)/pCR4-TOPO and the sequence was verified with T7 and T13-0 primers by Sanger sequencing (DNA Sequencing Core, MDACC). Then *NheI* and *SmaI* were used to digest ROR1R(CoOp)/pCR4-TOPO and CD19RCD28mZ(CoOp)/pEK plasmids and appropriate bands were purified by gel electrophoresis and ligated with T4 DNA Ligase to generate ROR1RCD28mZ(CoOp)/pEK. The ROR1-specific CAR was then transferred into a SB transposon by digestion of CD19RCD28mZ(CoOp)/pSBSO-MCS and ROR1RCD28mZ(CoOp)/pEK with *NheI* and *SpeI*, removal of phosphates by

*Antarctic Phosphatase* from pSBSO-MCS digestion, isolation of ROR1RCD28mZ and pSBSO-MCS bands by gel electrophoresis, and ligation with T4 DNA Ligase to generate ROR1RCD28mZ(CoOp)/pSBSO-MCS. The final ROR1RCD28 transposon plasmid was constructed by digesting CD19RCD28mZ(CoOp)/pSBSO-SIM with *NheI*, *XmaI*, and *Antarctic Phosphatase* and ROR1RCD28mZ(CoOp)/pSBSO-MCS with *NheI*, *XmnI*, and *XmaI*, purifying appropriate bands by gel electrophoresis, and ligating them together with T4 DNA Ligase to generate ROR1RCD28CD3z/pSBSO-SIM plasmid. Similarly, the final ROR1RCD137 transposon plasmid was constructed by digesting CD19R-CD28Tm-41BBCyt-Z(CoOp)/pSBSO-FRA with *NheI*, *XmaI*, and *Antarctic Phosphatase* and ROR1RCD28mZ(CoOp)/pSBSO-MCS with *NheI*, *XmnI*, and *XmaI*, purifying appropriate bands by gel electrophoresis, and ligating them together with T4 DNA Ligase to generate ROR1RCD137CD3z/pSBSO-FRA plasmid. Identities of final ROR1R plasmids were distinguished from CD19R plamids by *PmlI* enzyme and pSBSO-SIM and pSBSO-FRA plasmids were distinguished by *BsrGI* enzyme (**Figure 7**).

## VI.B. Cell Culture

Three media formulations were used herein for tissue culture. First, RPMI-CM was composed of RPMI (Gibco, Grand Island, NY), 10% heat-inactivated fetal bovine serum (FBS; Hyclone, Logan, UT), and 1% Glutamax-100 (Gibco). Similarly, RPMI-NaPyr-CM was RPMI, 10% FBS, 1% sodium pyruvate solution (Gibco), and 1% Glutamax-100. Last, DMEM-CM was made with DMEM (Sigma, St. Louis, MO), 10%

FBS, 1% sodium pyruvate solution, and 1% Glutamax-100. All tissue culture work was performed with 5% $CO_2$ at 37°C in humidified conditions unless otherwise stated.

VI.B.1. Established Tumor Cell Lines

Jurkat, HCT-116, Kasumi3, and K562 cell lines were acquired from American Type Culture Collection (ATCC; Manassas, VA). K562-derived aAPC (clone #4 and clone#9) were acquired as previously described from the University of Pennslyvania courtesy of Dr. Carl June.(57, 275, 278, 279) B-cell acute lymphoblastic leukemia (B-ALL) cell lines cALL2, Kasumi2, REH, and RCH-ACV cell lines were gifts from Dr. Jeff Tyner (OHSU), pancreatic cancer cell lines (BxPC3, CaPan2, MiaPaCa2, and Su8686) were donated by Dr. Viji Ramachandran (MDACC), and ovarian cancer cell lines (A2780, CAOV3, EFO21, Hey, IGROV1, OC314, OVCAR3, and UPN251) were provided by Dr. Robert Bast (MDACC). Cell cultures were maintained in (i) RPMI-CM: K562 parental cells, clone#1 aAPC, clone#4 aAPC, clone A6 aAPC, clone A3 aAPC, clone D4 aAPC, Jurkat, cALL2, Kasumi2, REH, RCH-ACV, and Kasumi3, (ii) RPMI-NaPyr-CM: A2780, EFO21, EFO27, Hey, IGROV1, OC314, OVCAR3, SKOV3, and UPN251, or (iii) DMEM-CM: CAOV3, BxPC3, CaPan2, MiaPaCa2, and Su8686. UPN251 cells were supplemented with insulin-transferrin-selenium solution (Gibco). Identities of all cell lines were confirmed by STR DNA Fingerprinting at MDACC's Cancer Center Support Grant (CCGS) supported facility "Characterized Cell Line Core."

VI.B.2. Genetic Modification of Cell Lines

*VI.B.2.a. ROR1 aAPC (clone#1)*

Clone#9 aAPC was generated though enforced expression of CD19, CD64, CD86, and CD137L on K562 cells (June CH, UPenn). This aAPC was further modified to express IL15/IL15Rα fusion protein (**Chapter VI.A.2.b**) on their surfaces and was sub-cloned to generate clone#27. Then clone#27 was made to express dROR1 (**Chapter VI.A.1.a**), and single cell clones were isolated based on expression of ROR1, CD137L, and IL15. The clone#1 aAPC uniformly expressed CD19, CD32, CD64, CD86, CD137L, IL15, and ROR1 and was cleared for co-culture following negative testing for mycoplasma and other microbial pathogens.

*VI.B.2.b. HLA$^{-/-}$ aAPC*

Zinc-finger nuclease (ZFN) specific for HLA-C was used to remove all MHC Class I expression from K562 cell surface (Torakai H, Lee DA, Rosoff H, and Cooper LJN). Clone#4 aAPC expresses IL15, CD86, and CD137L, so in order to investigate the roles of these molecules on γδ T cell proliferation new aAPC were constructed on K562 background (**Figure 28**). SB transposon containing IL15/IL15Rα fusion protein and SB11 transposase were electro-transferred into K562 cells (CD86$^{neg}$ and CD137L$^{neg}$) using Amaxa nucleofection and Kit V (cat#VCA-1003, Lonza, Basel, Switzerland). FACS was used to isolate IL15$^+$ cells, which were electroporated with SB11 and SB transposons containing either CD86 or CD137L. Cells were sorted again by FACS to

obtain IL15$^+$CD86$^+$ or IL15$^+$CD137L$^+$ as single cell clones A3 (IL15$^+$CD86$^+$CD137L$^{neg}$) and D4 (IL15$^+$CD86$^{neg}$CD137L$^+$), respectively. Single cell sorting FACS was also used to make a single cell clone (A6; IL15$^+$CD86$^{neg}$CD137L$^{neg}$) of cells electroporated once. Each cell line was negative for mycoplasma and microbial pathogens.

*VI.B.2.c. Lenitviral Packaging and Gene Transduction*

Lentivirus particles were packaged according to a modified version of a protocol described elsewhere.(357) Briefly, packaging cells (293-METR) were plated on flasks and transfected the following day with pCMV R8.2, VSV-G, and pLVU3G-effLuc-T2A-mKateS158A (**Figure 45**) plasmids in conjunction with Lipofectamine 2000 transfection reagent according to manufacturer's instructions (Invitrogen). Virus-like particles were harvested 48 and 72 hours post-transfection and were concentrated on 100 kDa NMWL filters (cat#UFC810096, MilliPore, Billerica, MA). CAOV3 cells were plated on wells of a 6 well plate, and the following day *ffluc*-mKate virus particles were added with 8 μg/ml polybrene then plate was spun at 1,800 rpm for 1.5 hours. The same was done for Kasumi2, except that polybrene was not added. Six hours later, the viral-conditioned supernatant was removed and the tissue culture media was immediately changed and changed the following day. Transduced CAOV3 were sub-cultured and single-cell clones were derived from limiting dilution that displayed the same morphology as the parental cell line and had uniform mKate fluorescence with high ($>10^6$ signal to noise ratio) *ffLuc* activity. CAOV3 clone 1C2 was used for mouse

experiments. Kasumi2 were sorted for mKate and were used as a bulk population for mouse experiments (**Figure 17a and 17b**).
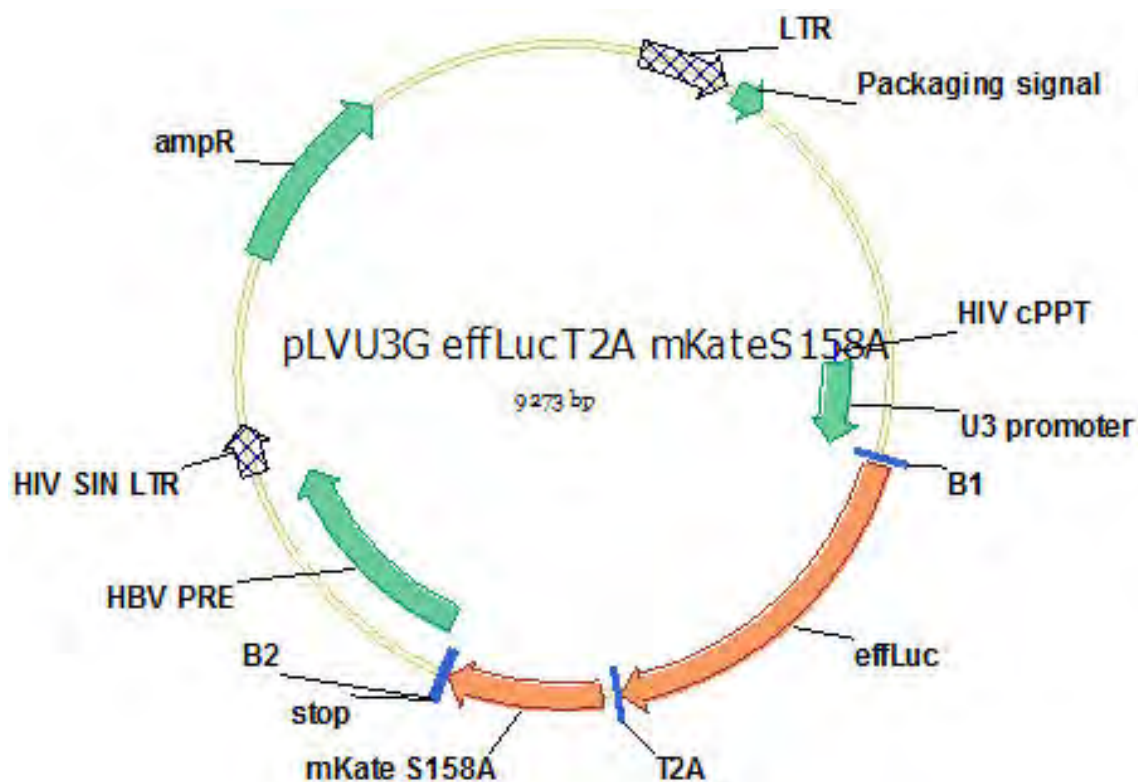
**Figure 45. DNA Plasmid Map for pLVU3G-effLuc-T2A-mKateS158A.** Annotations are, LTR: long terminal repeat; HIV cPPT: HIV central polypurine tract; B1: Gateway donor site B1; effLuc: enhanced *firefly Luciferase*; T2A: T2A ribosomal slip site; mKate S158A: enhanced mKate red fluorescence protein; B2: Gateway donor site B2; HBV PRE: Hepatitis B post-translational regulatory element; HIV SIN LTR: HIV self-inactivating long terminal repeat; ampR: ampicillin resistance (β-Lactamase).

VI.B.3. Primary Tumor Cells

PBMC were isolated by Ficoll-Hypaque (GE Healthcare) from patients with CLL diagnosis after informed consent was granted. Samples were cryopreserved and were thawed and used the day of the experiments. All cells frozen at the Cooper Lab were cryopreserved in 50% FBS, 40% RPMI, 10% dimethylsulfoxide (DMSO) termed "free media." All patient samples were maintained in RPMI-CM.

VI.B.4. Lymphocyte Cultures

All PBMC from adult donor blood or UCB used in this dissertation were obtained after informed consent and were isolated from whole blood by Ficoll-Hypaque or steady-state apheresis. PBMC were cryopreserved and thawed for experimental use whereas UCB were freshly isolated and immediately used. All aAPC were γ-irradiated (100 Gy) prior to co-culture and were then used immediately or were cryopreserved then thawed at the time of the co-culture. Validation of co-expression of cell surface markers (for example CD19, CD64, CD86, CD137L, and IL15 (co-expressed with eGFP) for clone #4) were performed before addition to T-cell cultures. All lymphocyte cultures were maintained in RPMI-CM.

*VI.B.4.a. CAR$^{neg}$ αβ T cells*

γ-irradiated clone#4 aAPC were loaded with OKT3 antibody, which is agonistic for CD3 thereby leading to T cell proliferation independent of the TCR specificity, by

OKT3 antibody docking on CD64 (high-affinity Fc Receptor) expressed on aAPC. $CD3^+$ T cells were stimulated with an equal number of OKT3-loaded clone#4 cells in the presence of exogenous IL2 (50 U/mL; Aldeleukin; Novartis, Switzerland) and IL21 (30 ng/mL; cat#AF20021; Peprotech, Rocky Hill, NJ) unless otherwise stated. Exogenous IL2 and IL21 were added back to cultures every 2-3 days along with at least half of the current volume of RPMI-CM.

*VI.B.4.b. CAR$^+$ $\alpha\beta$ T cells*

$CAR^+$ T cells were propagated based on modified standard operating protocols as previously described.(57, 273) Cryopreserved PBMC were thawed the day of the electroporation (designated day 0) and rested for 2 hours in RPMI-CM at 37$^o$C. Cells for electroporation were spun at 200g for 10 minutes, enumerated, and $2x10^7$ cells were mixed with DNA (5 μg SB11 transposase and 15 μg SB transposon) in Human T cell Nucleofector Solution (cat#VPA-1002, Lonza) then added to a cuvette, which was then electroporated on the U-014 program of Amaxa Nucleofector II (Lonza). Transfected cells were then added to wells of a 6-well plate containing phenol-free RPMI, 20% FBS, and 1x Glutamax-100. The following day, electroporated T cells were phenotyped and stimulated with aAPC according to their CAR expression. A ratio of 2:1 of clone#4 to CD19-specific $CAR^+$ T cells was used and a 1:1 ratio of clone#1 to ROR1-specific $CAR^+$ T cells was used. Each co-culture was supplemented with IL21 during the first week (given every 2-3 days) and with both IL2 and IL21 for the subsequent weeks. CAR expression was evaluated each week in order to do the stimulation according to

CAR$^+$ T cells. If NK cells reached >10% of the total populations, they were depleted from co-cultures with paramagnetic CD56 microbeads (cat#130-050-401, Miltenyi Biotec, Auburn, CA) and LS columns (cat#130-042-401, Miltenyi Biotec). Stocks were made of CAR$^+$ T cells at days 14, 21, 28, and 35 (where applicable), and inferred cell numbers were calculated by the number of cells that were generated multiplied by the fold change from the previous week relative to the number of cells that were carried forward. Phenotyping and functional analyses were performed between days 21 – 28 unless otherwise stated. For ROR1-specific CAR$^+$ T cell studies, 3 normal donors were tested in 3 independent experiments.

*VI.B.4.c. CAR$^+$ $\gamma\delta$ T cells*

CAR$^+$ $\gamma\delta$ T cells were generated as previously described.(311) Briefly, $10^8$ PBMC were electroporated as described above for CAR$^+$ $\alpha\beta$ T cells (**Chapter VI.B.4.b.**), and were then sorted for $\gamma\delta$ T cells using TCR$\gamma/\delta$+ Isolation Kit (cat#130-092-892, Miltenyi Biotec). Co-cultures were established with clone#4 along with IL2 and IL21 from the start of the cultures where cytokines were added every 2-3 days and clone#4 aAPC was added every 7 days at a 2:1 ratio with CAR$^+$ $\gamma\delta$ T cells. NK cells were depleted from co-cultures when they reached >10% of total cells as described above. T cells were phenotyped for CD3, Fc (CAR), CD56, and TCR$\gamma\delta$ every week to monitor the co-cultures. Cells were cryopreserved at days 21, 28, and 35 and inferred cell numbers were calculated as described above. Functional assays were performed between the

third and fifth weeks of stimulation. Six donors were tested in 3 independent experiments.

*VI.B.4.d. Polyclonal γδ T cells*

Experiments were initiated to expand γδ T cells on aAPC that did not express a CAR. Thawed PBMC ($10^8$) were depleted of NK cells as described above and were then labeled with TCRγ/δ+ T-cell isolation kit and placed on LS columns to separate γδ T cells in the unlabeled fraction from other cells attached to magnet. γδ T cells were stimulated at a ratio of one T cell to two aAPC (clone #4) in presence of exogenous IL2 and IL21. Cells were serially re-stimulated with addition of aAPC every 7 days for three weeks. FACS was used to isolate Vδ1 (TCRδ1$^+$ TCRδ2$^{neg}$), Vδ2 (TCRδ1$^{neg}$ TCRδ2$^+$), and Vδ3 (TCRδ1$^{neg}$ TCRδ2$^{neg}$) populations, which were stimulated as above with clone#4 aAPC twice and then phenotyped and used for functional assays. UCB-derived mononuclear cells were isolated from fresh Ficoll-Hypaque gradients by FACS following staining for TCRγδ and CD3, and were stimulated for five weeks on aAPC as per PBMC. Ten PBMC donors were tested in six independent experiments and five UCB donors were tested in four independent experiments. Four donors were tested in 2 independent experiments for Vδ sorting assays.

*VI.B.4.e. NK cells*

As controls for killing and allogeneic reactivity, NK cells autologous to γδ T cells were separated from healthy donor PBMC with CD56 microbeads and LS columns and were then stimulated at a 1:2 ratio with clone#4 aAPC in cultures that were supplemented at the initiation of culture and every 2-3 days later with IL2 and IL21.

*VI.B.4.f. γδ T cell Proliferation in Hypoxia*

A dedicated incubator set to 1% $O_2$, 5% $CO_2$, and $37^oC$ under humidified conditions was used to assess proliferation in hypoxia in parallel to "normal" tissue culture incubators set at 20% $O_2$, 5% $CO_2$, and $37^oC$ under humidified conditions. Parallel co-cultures were added to the incubators and were analyzed after the reported times.

VI.B.5 γδ T cell Co-culture Deconstruction

Experiments were implemented to assess the relative contribution of co-culture molecules to γδ T cell proliferation. This was dissected by cytokine dependence and dependence upon molecules on the aAPC using new aAPC described in **Chapter VI.B.2.b.** (**Figure 28**).

*VI.B.5.a. Effects of Cytokines on γδ T cell Proliferation*

In order to assess the dependence of γδ T cells on cytokines for proliferation, co-cultures were initiated with $10^5$ γδ T cells and $2x10^5$ clone#4 aAPC and then were added to an equal volume of (i) complete media (CM), (ii) CM and 100 U/mL IL2, (iii) CM and 60 ng/mL IL21, or (iv) CM, 100 U/mL IL2, and 60 ng/mL IL21. Co-cultures were counted 9 days after initiation to determine yields. Three donors were tested in two independent experiments.

*VI.B.5.b. Effects of Co-Stimulation on γδ T cell Proliferation*

HLA$^{-/-}$ aAPC (**Chapter VI.B.2.b**) were used to assess effects of co-stimulation on γδ T cell growth. Co-cultures were then initiated with $10^5$ γδ T cells in CM, 100 U/mL IL2, and 60 ng/mL IL21 and were added to $2x10^5$ γ-irradiated (i) parental K562 cells, (ii) clone A6, (iii) clone A3, (iv) clone D4, (v) clone#4 aAPC, or (vi) no aAPC. Co-cultures were counted 9 days as above with cytokine experiments. Three donors were tested in two independent experiments.

**VI.C. Multiplex Gene Expression Analysis**

At Day 22 of co-culture on aAPC, $>10^5$ T cells were lysed at a ratio of 5 μL RLT Buffer (Qiagen) per $3x10^4$ cells and frozen at -80°C in replicate vials for one time use. RNA lysates were thawed and immediately analyzed using nCounter Analysis System

(NanoString Technologies, Seattle, WA) with "designer TCR expression array" (DTEA) as previously described or with "lymphocyte codeset array" (LCA; **Appendix A**).(290, 311) DTEA data was normalized to both spike positive control RNA and housekeeping genes (ACTB, G6PD, OAZ1, POLR1B, POLR2A, RPL27, Rps13, and TBP) where 2 normalization factors were calculated and applied to the raw coutns. Each normalization factor was calculated from the average of sums for all samples divided by the sum of counts for an individual sample. Reported expression of TCR frequencies for ROR1-specific T cells (**Figure 14**) was calculated as counts for each TCRα or TCRβ allele over the total sum of TCRα or TCRβ counts, respectively. Total counts for LCA genes described in ROR1-specific CAR$^+$ T cells (**Figures 12 and 13**) and for TCRδ and TCRγ alleles in polyclonal γδ T cells were directly reported as normalized counts (**Figure 30**). For Vδ sorted γδ T cells, the normalized counts were reported at frequencies of each Vδ population per donor for each TCRδ or TCRγ allele (**Figure 32**). For example, %Vδ1*01 = (Vδ1*01)$_{Vδ1}$ / [(Vd1*01)$_{Vδ1}$ + (Vδ1*01)$_{Vδ2}$ + (Vδ1*01)$_{Vδ3}$].

### VI.D. Immunostaining

Antibodies directly conjugated to FITC, PerCP/Cy5.5, PE, and APC were used at 1:20, 1:33, 1:40, and 1:40 dilutions, respectively, in 100 μL FACS buffer (PBS, 0.1% FBS, 0.1% sodium azide) unless otherwise stated. A complete list of antibodies, clonotypes, and vendors can be found in **Appendix B**. CAR detection was primarily performed with anti-human Fc antibody (Invitrogen). CD19-specific CAR was stained with an idiotypic

antibody conjugated to AlexFluor-647 in some instances.(259) BD FACS CAlibur was used for most flow cytometry. Samples were analyzed with FlowJo software (version 7.6.5). BD FACS Aria Ilu II was used to sort cells where appropriate and was used to isolate single cell clones in 96 well plates for aAPC cloning strategies. Tumor cells transduced with *ffLuc*-mKate lentivirus particles were sorted for mKate expression on BD Influx for bulk populations or as single cell clones as appropriate.

## VI.E. Cytokine Production

Expression of cytokines was assessed by intracellular cytokine staining (ICS) and secretion of cytokines into tissue culture supernatants was evaluated by Luminex multiplex analysis. Co-cultures were set up with T cells and targets as described for each experiment and were incubated at $37^{o}$C. For ICS, Brefeldin-A (GolgiPlug; BD Biosciences) was added to co-cultures to block exocytosis and secretion of cytokines produced in response to agonists. All ICS experiments were incubated for 6 hours and were then (i) stained for surface markers, e.g. CD3 and CAR, (ii) fixed and permeabilized with BD Cytofix/Cytoperm (BD Biosciences), (iii) stained for intracellular proteins, e.g. IFNγ and TNFα, and (iv) analyzed by flow cytometry. Co-cultures to assess cytokine secretion were incubated for 24 hours and supernatants from triplicate wells were pooled and analyzed by Bio-Plex Human Cytokine Group I 27-plex Assay (#L50-0KCAF0Y, BioRad Technologies, Hercules, CA) using Luminex100 (xMap Technologies, Austin, TX).

**VI.F. *In Vitro* Killing Assays**

VI.F.1. Chromium Release Assay

*In vitro* specific lysis was assessed using a standard 4-hour CRA, as previously described.(57) Purified antibodies specific for NKG2D (clone 1D11; BD Biosciences), DNAM1 (clone DX11; BD Biosciences), TCRγδ (clone B1; BD Biosciences), and TCRγδ (clone IMMU510; Thermo Fisher, Pittsburg, PA) were used for neutralization experiments at 0.3, 1.0, and 3.0 μg/mL final concentrations in CRA at E:T ratios of 12:1. Normal mouse serum was used as a negative control at the same concentrations.

VI.F.2. Long-term Killing Assay

Tumor cells were seeded in wells of 12-well plates at a density of $4x10^4$ cells/well. The following day, $5x10^5$ γδ T cells were added to each well of the plate and an equal number was added to a well without tumor cells (media only). One well of tumor cells had an equal volume of RPMI-CM added as a positive control for growth. After 2 days, supernatants were harvested, wells were washed in PBS, and remaining tumor cells were harvested with trypsin-EDTA and were then enumerated. The frequencies of cells remaining were normalized to mock treated tumor cells.

**VI.G. Mixed Lymphocyte Reactions**

B cells from healthy donors were isolated with CD19 microbeads (cat#130-050-301, Miltenyi Biotec) the day of each assay and were used as target cells in proliferation, IFNγ production (ELISpot), and cytolysis assays. Standard 4-hour CRA were used for the latter as described above. For proliferation assays, effector cells were labeled with PKH26 red fluorescent dye according to manufacturer's instructions (Sigma) and were co-cultured with target cells for 4 days at $37^{o}$C at an E:T ratio of 5:1. Co-cultures were stained for CD3, CD19, and CD56 then were analyzed by flow cytometry. Similarly, IFNγ ELISpot plate (Mabtech, Mariemont, OH) was set up with effector (γδ T cells) and target (B cells) at an E:T ratio of 0.3:1 and plate was incubated for 24 hours at $37^{o}$C then stained according to manufacturer's instructions, and spots were counted on Immunospot (CTL, Shaker Heights, OH). OKT3-loaded aAPC were used as positive controls and mock treated were used as negative controls along with autologous B cells. Co-cultures of effectors and allogeneic PBMC (normalized to equal CD34$^{+}$ cells) at a 4:1 ratio of effectors to CD34$^{+}$ HSC were incubated at $37^{o}$C for 4 hours and were then plated in wells of 6-well plates in semi-solid HSC-CFU Complete without EPO (Miltenyi Biotec). After 12 days, individual colonies were counted under inverted microscope. Colonies formed with effectors alone or targets alone were used to normalize the relative number of colonies formed for each donor.

**VI.H. *In Vivo* Anti-tumor Activity**

*In vivo* anti-tumor efficacy was assessed in NSG mice (NOD.Cg-Prkdc$^{scid}$ Il2rγ$^{tm1Wjl}$/SzJ; Jackson Laboratories). Non-invasive BLI was performed during the course of the experiments to measure tumor burden of cell lines expressing *ffLuc* following subcutaneous D-Luciferin (cat#122796, Caliper, Hopkinton, MA) administration with IVIS-100 Imager (Caliper). BLI was analyzed using Living Image software (version 2.50, Xenogen, Caliper).

VI.H.1. ROR1-specific Anti-leukemia Effects

Kasumi-2-*ffLuc*-mKate cells (4x10$^4$ per mouse) were engrafted into NSG mice (n = 15) intravenously (i.v.) the day before the first T cell dose (designated Day -1). The following day (Day 0), treatment groups for mice with tumors were set up with (i) no treatment (n = 5), (ii) ROR1RCD28 T cells (n = 5), and (iii) ROR1RCD137 T cells (n = 5). Mice were injected with T cells only as controls for xenogeneic reactivity (one mouse per T cell type). T cell doses (10$^7$ total cells per mouse) were given on days 0, 7, and 14. Frequencies for CAR expression for ROR1RCD28 were 96%, 91%, and 90% and for ROR1RCD137 were 94%, 62%, and 46% on days 0, 7, and 14, respectively. Survival was the primary endpoint for the study and BLI from tumor *ffLuc* was monitored twice per week as above.

VI.H.2. CD19-specific Anti-leukemia Activity

The anti-tumor effects of CD19-specific CAR$^+$ γδ T cells were evaluated as previously described.(311)

VI.H.3. γδ T cells Clearance of Ovarian Cancer

CAOV3-*ffLuc*-mkate (clone 1C2; $3x10^6$ cells/mouse) tumors were established by intraperitoneal (i.p.) injection and mice were randomly distributed into treatment groups. Eight days later (designated Day 0), a dose escalation regimen was initiated with polyclonal γδ T cells administered i.p. and PBS administered i.p. as a negative control. T cell doses infused were $3x10^6$, $6x10^6$, $10^7$, and $1.5x10^7$ on days 0, 7, 14, and 21, respectively. BLI was monitored during the course of the experiment by weekly monitoring of tumor *ffLuc* activity as above. Survival was the primary endpoint for the experiment.

**APPENDICIES**

## Appendix A. Lymphocyte CodeSet Array

| GENE ID | Access-ion | Target Region | Target Sequence |
|---|---|---|---|
| ABCB1 | NM_000 927.3 | 3910-4010 | TATAGCACTAAAGTAGGAGACAAAGGAACTCAGCTCTCTGGTGGCCAGAAAC AACGCATTGCCATAGCTCGTGCCCTTGTTAGACAGCCTCATATTTTGC |
| ABCG2 | NM_004 827.2 | 285-385 | AGGATTTAGGAACGCACCGTGCACATGCTTGGTGGTCTTGTTAAGTGGAAACT GCTGCTTTAGAGTTTGTTTGGAAGGTCCGGGTGACTCATCCCAACAT |
| ACTB | NM_001 101.2 | 1010-1110 | TGCAGAAGGAGATCACTGCCCTGGCACCCAGCACAATGAAGATCAAGATCAT TGCTCCTCCTGAGCGCAAGTACTCCGTGTGGATCGGCGGCTCCATCCT |
| ADAM19 | NM_023 038.3 | 1690-1790 | GAGAAGGTGAATGTGGCAGGAGACACCTTTGGAAACTGTGGAAAGGACATG AATGGTGAACACAGGAAGTGCAACATGAGAGATGCGAAGTGTGGGAAGA |
| AGER | NM_001 136.3 | 340-440 | GAAAGGAGACCAAGTCCAACTACCGAGTCCGTGTCTACCAGATTCCTGGGAA GCCAGAAATTGTAGATTCTGCCTCTGAACTCACGGCTGGTGTTCCCAA |
| AHNAK | NM_001 620.1 | 15420-15520 | GGATTTGACCTGAATGTTCCTGGGGGGTGAAATTGATGCCAGCCTCAAGGCTCC GGATGTAGATGTCAACATCGCAGGGCCGGATGCTGCACTCAAAGTCG |
| AIF1 | NM_032 955.1 | 315-415 | AAAAGCGAGAGAAAAGGAAAAGCCAACAGGCCCCCCAGCCAAGAAAGCTAT CTCTGAGTTGCCCTGATTTGAAGGGAAAAGGGATGATGGGATTGAAGGG |
| AIM2 | NM_004 833.1 | 607-707 | ACGTGCTGCACCAAAAGTCTCTCCTCATGTTAAGCCTGAACAGAAACAGATG GTGGCCCAGCAGGAATCTATCAGAGAAGGGTTTCAGAAGCGCTGTTTG |
| AKT1 | NM_005 163.2 | 1772-1872 | TTCTTTGCCGGTATCGTGTGGCAGCACGTGTACGAGAAGAAGCTCAGCCCACC CTTCAAGCCCCAGGTCACGTCGGAGACTGACACCAGGTATTTTGATG |
| ALDH1A1 | NM_000 689.3 | 11-111 | ATTGCTGAGCCAGTCACCTGTGTTCCAGGAGCCGAATCAGAAATGTCATCCTC AGGCACGCCAGACTTACCTGTCCTACTCACCGATTTGAAGATTCAAT |
| ANXA1 | NM_000 700.1 | 515-615 | GAAATCAGAGACATTAACAGGGTCTACAGAGAGGAACTGAAGAGAGATCTG GCCAAAGACATAACCTCAGACACATCTGGAGATTTTCGGAACGCTTTGC |
| ANXA2P2 | NR_003 573.1 | 257-357 | ATATTGTCTTCTCCTACCAGAGAAGGACCAAAAAGGAACTTGCATCAGCACT GAAGTCAGCCTTATCTGGCCACCTGGAGACGGTGATTTTGGGCCTATT |
| AP1 | NM_002 228.3 | 140-240 | ACACAGCCAGCCAGCCAGGTCGGCAGTATAGTCCGAACTGCAAATCTTATTTT CTTTTCACCTTCTCTCTAACTGCCCAGAGCTAGCGCCTGTGGCTCCC |
| Apaf1 | NM_181 869.1 | 1160-1260 | TTCTGATGAAACTGCAGAATCTTTGCACACGGTTGGATCAGGATGAGAGTTTT TCCCAGAGGCTTCCACTTAATATTGAAGAGGCTAAAGACCGTCTCCG |
| ARG1 | NM_000 045.2 | 505-605 | AAGGAACTAAAAGGAAAGATTCCCGATGTGCCAGGATTCTCCTGGGTGACTC CCTGTATATCTGCCAAGGATATTGTGTATATTGGCTTGAGAGACGTGG |
| ATM | NM_000 051.3 | 30-130 | ACGCTAAGTCGCTGGCCATTGGTGGACATGGCGCAGGCGCGTTTGCTCCGAC GGGCCGAATGTTTTGGGGCAGTGTTTTGAGCGCGCGGAGACCGCGTGATA |
| ATP2B4 | NM_001 684.3 | 7640-7740 | CTTCCCATAGTATCATCTGTCCTCTGGAATGACTCTCCTGTCCCTAAAGGGGTT AAGAGAGAGATCACCTAGAAATCCCTCTGGACACTTGTGGGTTCTT |
| B2M | NM_004 048.2 | 25-125 | CGGGCATTCCTGAAGCTGACAGCATTCGGGCCGAGATGTCTCGCTCCGTGGCC TTAGCTGTGCTCGCGCTACTCTCTCTTTCTGGCCTGGAGGCTATCCA |
| BACH2 | NM_021 813.2 | 3395-3495 | TGTGGCACTGTTCATCTGCTGTCCCGAAGAAACCGAGAACACATTTGGTGCAC ACTACAGCGGTCTTAGCAGCAATACTGTTCCGAAGTATCCTCTCCTC |
| BAD | NM_004 | 195-295 | CAGCTGTGCCTTGACTACGTAACATCTTGTCCTCACAGCCCAGAGCATGTTCC |

| | | | |
|---|---|---|---|
| | 322.2 | | AGATCCCAGAGTTTGAGCCGAGTGAGCAGGAAGACTCCAGCTCTGCA |
| BATF | NM_006 399.3 | 825-925 | CACTGTGGGTTGCAGGCCCAATGCAGAAGAGTATTAAGAAAGATGCTCAAGT CCCATGGCACAGAGCAAGGCGGGCAGGGAACGGTTATTTTTCTAAATA |
| BAX | NM_138 761.2 | 694-794 | ATTTTTCTGGGAGGGGTGGGGATTGGGGGACATGGGCATTTTTCTTACTTTTG TAATTATTGGGGGGGTGTGGGGAAGAGTGGTCTTGAGGGGGTAATAAA |
| BCL10 | NM_003 921.2 | 1250-1350 | TGAAAATACCATCTTCTCTTCAACTACACTTCCCAGACCTGGGGACCCAGGGG CTCCTCCTTTGCCACCAGATCTACAGTTAGAAGAAGAAGGAACTTGT |
| Bcl2 | NM_000 633.2 | 1525-1625 | CCAAGCACCGCTTCGTGTGGCTCCACCTGGATGTTCTGTGCCTGTAAACATAG ATTCGCTTTCCATGTTGTTGGCCGGATCACCATCTGAAGAGCAGACG |
| BCL2L1 | NM_138 578.1 | 1560-1660 | CTAAGAGCCATTTAGGGGCCACTTTTGACTAGGGATTCAGGCTGCTTGGGATA AAGATGCAAGGACCAGGACTCCCTCCTCACCTCTGGACTGGCTAGAG |
| BCL2L11 | NM_138 621.2 | 2825-2925 | TGTTGGCACCAGAACTTAAAGCGATGACTGGATGTCTCTGTACTGTATGTATC TGGTTATCAAGATGCCTCTGTGCAGAAAGTATGCCTCCCGTGGGTAT |
| Bcl6 | NM_001 706.2 | 675-775 | GTTGTGGACACTTGCCGGAAGTTTATTAAGGCCAGTGAAGCAGAGATGGTTT CTGCCATCAAGCCTCCTCGTGAAGAGTTCCTCAACAGCCGGATGCTGA |
| Beta-arrestin (ARRB2 and ARRB2) | NM_004 313.3 | 1652-1752 | CATTAATTTTTTGACTGCAGCTCTGCTTCTCCAGCCCCGCCGTGGGTGGCAAG CTGTGTTCATACCTAAATTTTCTGGAAGGGGACAGTGAAAAGAGGAG |
| BHLHE41 | NM_030 762.2 | 655-755 | CGCCCATTCAGTCCGACTTGGATGCGTTCCACTCGGGATTTCAAACATGCGCC AAAGAAGTCTTGCAATACCTCTCCCGGTTTGAGAGCTGGACACCCAG |
| BID | NM_197 966.1 | 2095-2195 | GCTTAGCTTTAGAAACAGTGCAACACTGGTCTGCTGTTCCAGTGGTAAGCTAT GTCCCAGGAATCAGTTTAAAAGCACGACAGTGGATGCTGGGTCCATA |
| BIRC2 | NM_001 166.3 | 1760-1860 | TGGGATCCACCTCTAAGAATACGTCTCCAATGAGAAACAGTTTTGCACATTCA TTATCTCCCACCTTGGAACATAGTAGCTTGTTCAGTGGTTCTTACTC |
| BMI1 | NM_005 180.5 | 1145-1245 | CCTGGAGAAGGAATGGTCCACTTCCATTGAAATACAGAGTTCGACCTACTTGT AAAAGAATGAAGATCAGTCACCAGAGAGATGGACTGACAAATGCTGG |
| BNIP3 | NM_004 052.2 | 325-425 | CACCTCGCTCGCAGACACCACAAGATACCAACAGGGCTTCTGAAACAGATAC CCATAGCATTGGAGAGAAAAACAGCTCACAGTCTGAGGAAGATGATAT |
| C10RF24 | NM_052 966.2 | 3526-3626 | TGCCCAATAGATTCAAGAGAAGCTAAGCGGAAATGGAGGGTGGAAGGTGTG ATCTGTGGGACTGTCTGGGCCTGTTACTCATCCTGCTATCAATTTCTTA |
| C11ORF17 | NM_020 642.3 | 570-670 | GAACATCTCTAAGGACCTCTACATAGAAGTATATCCAGGGACCTATTCTGTCA CTGTGGGCTCAAATGACTTAACCAAGAAGACTCATGTGGTAGCAGTT |
| C5ORF13 | NM_001 142474.1 | 990-1090 | AAACTCATTGTTTCCTTGTGGTAAGTGACCGAGATGCTGCCACAGGACCTGAG ACACTGATGAATGGTGCTATTTTGGACTTTCAACATGCTCCTTGGCG |
| C80RF70 | NM_016 010.2 | 665-765 | ACGATTACCGCAGCCAAGTGGCGCTGGCAAAACTGTTGTAGGTGTTCCTTCAG GTAAAGTGTCTTCAAGTAGCAGCTCTTTGGGAAACAAACTTCAGACC |
| CA9 | NM_001 216.2 | 960-1060 | CAGGTCCCAGGACTGGACATATCTGCACTCCTGCCCTCTGACTTCAGCCGCTA CTTCCAATATGAGGGGTCTCTGACTACACCGCCCTGTGCCCAGGGTG |
| CASP1 | NM_033 292.2 | 575-675 | ACAGGCATGACAATGCTGCTACAAAATCTGGGGTACAGCGTAGATGTGAAAA AAAATCTCACTGCTTCGGACATGACTACAGAGCTGGAGGCATTTGCAC |
| Caspase 9 | NM_052 813.2 | 1850-1950 | CGCTGACTTGGCCTGGAACGAGGAATCTGGTGCCCTGAAAGGCCCAGCCGGA CTGCCGGGCATTGGGGCCGTTTGTTAAGCGGCACTCATTTTGCGGAGG |
| CAT | NM_001 752.2 | 1130-1230 | ATGCTTCAGGGCCGCCTTTTTGCCTATCCTGACACTCACCGCCATCGCCTGGG ACCCAATTATCTTCATATACCTGTGAACTGTCCCTACCGTGCTCGAG |
| CCL3 | NM_002 983.2 | 681-781 | CTGTGTAGGCAGTCATGGCACCAAAGCCACCAGACTGACAAATGTGTATCGG ATGCTTTTGTTCAGGGCTGTGATCGGCCTGGGGAAATAATAAAGATGC |

| | | | |
|---|---|---|---|
| CCL4 | NM_002984.2 | 35-135 | TTCTGCAGCCTCACCTCTGAGAAAACCTCTTTGCCACCAATACCATGAAGCTCTGCGTGACTGTCCTGTCTCTCCTCATGCTAGTAGCTGCCTTCTGCTC |
| CCL5 | NM_002985.2 | 280-380 | AGTGTGTGCCAACCCAGAGAAGAAATGGGTTCGGGAGTACATCAACTCTTTGGAGATGAGCTAGGATGGAGAGTCCTTGAACCTGAACTTACACAAATTT |
| CCNB1 | NM_031966.2 | 715-815 | AACTTGAGGAAGAGCAAGCAGTCAGACCAAAATACCTACTGGGTCGGGAAGTCACTGGAAACATGAGAGCCATCCTAATTGACTGGCTAGTACAGGTTCA |
| CCND1 | NM_053056.2 | 690-790 | TTGAACACTTCCTCTCCAAAATGCCAGAGGCGGAGGAGAACAAACAGATCATCCGCAAACACGCGCAGACCTTCGTTGCCCTCTGTGCCACAGATGTGAA |
| CCR1 | NM_001295.2 | 535-635 | CATCATTTGGGCCCTGGCCATCTTGGCTTCCATGCCAGGCTTATACTTTTCCAAGACCCAATGGGAATTCACTCACCACACCTGCAGCCTTCACTTTCCT |
| CCR2 | NM_001123041.2 | 20-120 | ACATTCTGTTGTGCTCATATCATGCAAATTATCACTAGTAGGAGAGCAGAGAGTGGAAATGTTCCAGGTATAAAGACCCACAAGATAAAGAAGCTCAGAG |
| CCR4 | NM_005508.4 | 35-135 | GGTCCTTCTTAGCATCGTGCTTCCTGAGCAAGCCTGGCATTGCCTCACAGACCTTCCTCAGAGCCGCTTTCAGAAAAGCAAGCTGCTTCTGGTTGGGCCC |
| CCR5 | NM_000579.1 | 2730-2830 | TAGGAACATACTTCAGCTCACACATGAGATCTAGGTGAGGATTGATTACCTAGTAGTCATTTCATGGGTTGTTGGGAGGATTCTATGAGGCAACCACAGG |
| CCR6 | NM_031409.2 | 935-1035 | CTTTAACTGCGGGATGCTGCTCCTGACTTGCATTAGCATGGACCGGTACATCGCCATTGTACAGGCGACTAAGTCATTCCGGCTCCGATCCAGAACACTA |
| CCR7 | NM_001838.2 | 1610-1710 | TTCCGAAAACCAGGCCTTATCTCCAAGACCAGAGATAGTGGGGAGACTTCTTGGCTTGGTGAGGAAAAGCGGACATCAGCTGGTCAAACAAACTCTCTGA |
| CD11b | NM_000632.3 | 515-615 | GCCCTCCGAGGGTGTCCTCAAGAGGATAGTGACATTGCCTTCTTGATTGATGGCTCTGGTAGCATCATCCCACATGACTTTCGGCGGATGAAGGAGTTTG |
| CD16 | NM_000570.3 | 73-173 | CCTATTCCTGTTCTATGGTGGGGCTCCATTGCGAGACTTCAGATTGAGAAATCAGATGAAGTTTCAAGAAAAGGAAACTGGCAGGTGACAGAGATGGGTG |
| CD160 | NM_007053.2 | 500-600 | TTGATGTTCACCATAAGCCAAGTCACACCGTTGCACAGTGGGACCTACCAGTGTTGTGCCAGAAGCCAGAAGTCAGGTATCCGCCTTCAGGGCCATTTTT |
| CD19 | NM_001770.4 | 1770-1870 | AGATTCACACCTGACTCTGAAATCTGAAGACCTCGAGCAGATGATGCCAACCTCTGGAGCAATGTTGCTTAGGATGTGTGCATGTGTGTAAGTGTGTGTG |
| CD19RCD28CAR | MDA_00002.1 | 2-102 | CAGGTGTTCCTGAAGATGAACAGCCTGCAGACCGACGACACCGCCATCTACTACTGTGCCAAGCACTACTACTACGGCGGCAGCTACGCCATGGACTACT |
| CD2 | NM_001767.2 | 1400-1500 | TGGGTCTCACTACAAGCAGCCTATCTGCTTAAGAGACTCTGGAGTTTCTTATGTGCCCTGGTGGACACTTGCCCACCATCCTGTGAGTAAAAGTGAAATA |
| CD244 | NM_016382.2 | 1150-1250 | AAGAGGAACCACAGCCCTTCCTTCAATAGCACTATCTATGAAGTGATTGGAAAGAGTCAACCTAAAGCCCAGAACCCTGCTCGATTGAGCCGCAAAGAGC |
| CD247 | NM_198053.1 | 1490-1590 | TGGCAGGACAGGAAAAAACCCGTCAATGTACTAGGATACTGCTGCGTCATTACAGGGCACAGGCCATGGATGGAAAACGCTCTCTGCTCTGCTTTTTTTCT |
| CD274 | NM_014143.2 | 684-784 | TAGGAGATTAGATCCTGAGGAAAACCATACAGCTGAATTGGTCATCCCAGAACTACCTCTGGCACATCCTCCAAATGAAAGGACTCACTTGGTAATTCTG |
| CD276 | NM_001024736.1 | 2120-2220 | ACATTTCTTAGGGACACAGTACACTGACCACATCACCACCCTCTTCTTCCAGTGCTGCGTGGACCATCTGGCTGCCTTTTTTCTCCAAAAGATGCAATAT |
| CD28 | NM_006139.1 | 305-405 | GCTTGTAGCGTACGACAATGCGGTCAACCTTAGCTGCAAGTATTCCTACAATCTCTTCTCAAGGGAGTTCCGGGCATCCCTTCACAAAGGACTGGATAGT |
| CD38 | NM_001775.2 | 1035-1135 | CCTTGACTCCTTGTGGTTTATGTCATCATACATGACTCAGCATACCTGCTGGTGCAGAGCTGAAGATTTTGGAGGGTCCTCCACAATAAGGTCAATGCCA |
| CD3D | NM_000732.4 | 110-210 | TATCTACTGGATGAGTTCCGCTGGGAGATGGAACATAGCACGTTTCTCTCTGGCCTGGTACTGGCTACCCTTCTCTCGCAAGTGAGCCCCTTCAAGATAC |

| Gene | Accession | Range | Sequence |
|---|---|---|---|
| CD3E | NM_000733.2 | 75-175 | AAGTAACAGTCCCATGAAACAAAGATGCAGTCGGGCACTCACTGGAGAGTTC TGGGCCTCTGCCTCTTATCAGTTGGCGTTTGGGGGCAAGATGGTAATG |
| CD4 | NM_000616.3 | 835-935 | AGACATCGTGGTGCTAGCTTTCCAGAAGGCCTCCAGCATAGTCTATAAGAAA GAGGGGGAACAGGTGGAGTTCTCCTTCCCACTCGCCTTTACAGTTGAA |
| CD40LG | NM_000074.2 | 1225-1325 | GCATTTGATTTATCAGTGAAGATGCAGAAGGGAAATGGGGAGCCTCAGCTCA CATTCAGTTATGGTTGACTCTGGGTTCCTATGGCCTTGTTGGAGGGGG |
| CD44 | NM_000610.3 | 2460-2560 | GTGGGCAGAAGAAAAAGCTAGTGATCAACAGTGGCAATGGAGCTGTGGAGG ACAGAAAGCCAAGTGGACTCAACGGAGAGGCCAGCAAGTCTCAGGAAAT |
| CD58 | NM_001779.2 | 478-578 | GTGCTTGAGTCTCTTCCATCTCCCACACTAACTTGTGCATTGACTAATGGAAG CATTGAAGTCCAATGCATGATACCAGAGCATTACAACAGCCATCGAG |
| CD63 | NM_001780.4 | 350-450 | GTCATCATCGCAGTGGGTGTCTTCCTCTTCCTGGTGGCTTTTGTGGGCTGCTGC GGGGCCTGCAAGGAGAACTATTGTCTTATGATCACGTTTGCCATCT |
| CD69 | NM_001781.1 | 460-560 | AGGACATGAACTTTCTAAAACGATACGCAGGTAGAGAGGAACACTGGGTTGG ACTGAAAAAGGAACCTGGTCACCCATGGAAGTGGTCAAATGGCAAAGA |
| CD80 | NM_005191.3 | 1288-1388 | AAAGATCTGAAGGTCCCACCTCCATTTGCAATTGACCTCTTCTGGGAACTTCC TCAGATGGACAAGATTACCCCACCTTGCCCTTTACGTATCTGCTCTT |
| CD86 | NM_006889.3 | 146-246 | TATGGGACTGAGTAACATTCTCTTTGTGATGGCCTTCCTGCTCTCTGGTGCTGC TCCTCTGAAGATTCAAGCTTATTTCAATGAGACTGCAGACCTGCCA |
| CD8A | NM_001768.5 | 1320-1420 | GCTCAGGGCTCTTTCCTCCACACCATTCAGGTCTTTCTTTCCGAGGCCCCTGTC TCAGGGTGAGGTGCTTGAGTCTCCAACGGCAAGGGAACAAGTACTT |
| CDH1 | NM_004360.2 | 1230-1330 | CGATAATCCTCCGATCTTCAATCCCACCACGTACAAGGGTCAGGTGCCTGAGA ACGAGGCTAACGTCGTAATCACCACACTGAAAGTGACTGATGCTGAT |
| CDK2 | NM_001798.2 | 220-320 | TCGCTGGCGCTTCATGGAGAACTTCCAAAAGGTGGAAAAGATCGGAGAGGGC ACGTACGGAGTTGTGTACAAAGCCAGAAACAAGTTGACGGGAGAGGTG |
| CDK4 | NM_000075.2 | 1055-1155 | ACTTTTAACCCACACAAGCGAATCTCTGCCTTTCGAGCTCTGCAGCACTCTTA TCTACATAAGGATGAAGGTAATCCGGAGTGAGCAATGGAGTGGCTGC |
| CDKN1A | NM_000389.2 | 1975-2075 | CATGTGTCCTGGTTCCCGTTTCTCCACCTAGACTGTAAACCTCTCGAGGGCAG GGACCACACCCTGTACTGTTCTGTGTCTTTCACAGCTCCTCCCACAA |
| CDKN1B | NM_004064.2 | 365-465 | GCTTCCGAGAGGGGGTTCGGGCCGCGTAGGGGCGCTTTGTTTTGTTCGGTTTTG TTTTTTTGAGAGTGCGAGAGAGGCGGTCGTGCAGACCCGGGAGAAAG |
| CDKN2C | NM_001262.2 | 1295-1395 | ATAATGTAAACGTCAATGCACAAAATGGATTTGGAAGGACTGCGCTGCAGGT TATGAAACTTGGAAATCCCGAGATTGCCAGGAGACTGCTACTTAGAGG |
| CEBPA | NM_004364.2 | 1320-1420 | GAGCTGGGAGCCCGGCAACTCTAGTATTTAGGATAACCTTGTGCCTTGGAAAT GCAAACTCACCGCTCCAATGCCTACTGAGTAGGGGGAGCAAATCGTG |
| CFLAR | NM_003879.3 | 445-545 | CAAGACCCTTGTGAGCTTCCCTAGTCTAAGAGTAGGATGTCTGCTGAAGTCAT CCATCAGGTTGAAGAAGCACTTGATACAGATGAGAAGGAGATGCTGC |
| CIITA | NM_000246.3 | 470-570 | GCCTGAGCAAGGACATTTTCAAGCACATAGGACCAGATGAAGTGATCGGTGA GAGTATGGAGATGCCAGCAGAAGTTGGGCAGAAAAGTCAGAAAAGACC |
| CITED2 | NM_006079.3 | 965-1065 | AGGAGCTGCCCGAACTCTGGCTGGGGCAAAACGAGTTTGATTTTATGACGGA CTTCGTGTGCAAACAGCAGCCCAGCAGAGTGAGCTGTTGACTCGATCG |
| CLA | NM_003006.3 | 2297-2397 | CATGGGCTGTTAGGTTGACTTCAGTTTTGCCTCTTGGACAACAGGGGGTCTTG TACATCCTTGGGTGACCAGGAAAAGTTCAGGCTATGGGGGGCCAAAG |
| CLIC1 | NM_001288.4 | 310-410 | GTGATGGGGCCAAGATTGGGAACTGCCCATTCTCCCAGAGACTGTTCATGGT ACTGTGGCTCAAGGGAGTCACCTTCAATGTTACCACCGTTGACACCAA |
| CMRF-35H | NM_007261.2 | 0-100 | CGGGGAAGTGAGAGTCGGGGATCAGTCCTGCAAGCTACGGAGTCACTACAGG GAGAGGTCTCATCACTAGAAATAGCCGAAGAACCTGCAGCCTCAACCA |

198

| CREB1 | NM_004 379.3 | 4855-4955 | TTTGATGGTAGGTCAGCAGCAGTGCTAGTCTCTGAAAGCACAATACCAGTCA GGCAGCCTATCCCATCAGATGTCATCTGGCTGAAGTTTATCTCTGTCT |
|---|---|---|---|
| CRIP1 | NM_001 311.4 | 269-369 | CAACCACCCCTGCTACGCAGCCATGTTTGGGCCTAAAGGCTTTGGGCGGGGC GGAGCCGAGAGCCACACTTTCAAGTAAACCAGGTGGTGGAGACCCCAT |
| CSAD | NM_015 989.4 | 205-305 | TCAAATTCTTCTGCCTAGCCTTAGCCATTAGAGAGAGGTCCTGCTAAAGATGG ACTGCAAATGCGCTTGATGGAAGGAGATGTCAATTCCACTGAAGTCC |
| CSF2 | NM_000 758.2 | 475-575 | AGATGAGGCTGGCCAAGCCGGGGAGCTGCTCTCTCATGAAACAAGAGCTAGA AACTCAGGATGGTCATCTTGGAGGGACCAAGGGGTGGGCCACAGCCAT |
| CSNK2A1 | NM_177 559.2 | 1930-2030 | CCATTCCCACCATTGTTCCTCCACCGTCCCACACTTTAGGGGGTTGGTATCTCG TGCTCTTCTCCAGAGATTACAAAAATGTAGCTTCTCAGGGGAGGCA |
| CTGF | NM_001 901.2 | 1100-1200 | ACCACCCTGCCGGTGGAGTTCAAGTGCCCTGACGGCGAGGTCATGAAGAAGA ACATGATGTTCATCAAGACCTGTGCCTGCCATTACAACTGTCCCGGAG |
| CTLA4 | NM_005 214.3 | 405-505 | AGTCTGTGCGGCAACCTACATGATGGGGAATGAGTTGACCTTCCTAGATGATT CCATCTGCACGGGCACCTCCAGTGGAAATCAAGTGAACCTCACTATC |
| CTNNA1 | NM_001 903.2 | 75-175 | TCGCCCAGCTAGCCGCAGAAATGACTGCTGTCCATGCAGGCAACATAAACTT CAAGTGGGATCCTAAAAGTCTAGAGATCAGGACTCTGGCAGTTGAGAG |
| CTNNB1 | NM_001 098210.1 | 1815-1915 | TCTTGCCCTTTGTCCCGCAAATCATGCACCTTTGCGTGAGCAGGGTGCCATTC CACGACTAGTTCAGTTGCTTGTTCGTGCACATCAGGATACCCAGCGC |
| CTNNBL1 | NM_030 877.3 | 855-955 | TGATGCCAACAAACTGTATTGCAGTGAAGTGCTGGCCATATTGCTCCAGGAC AATGATGAAAACAGGGAATTGCTTGGGGAGCTGGATGGAATCGATGTG |
| CX3C1 | NM_002 996.3 | 140-240 | AGCACCACGGTGTGACGAAATGCAACATCACGTGCAGCAAGATGACATCAAA GATACCTGTAGCTTTGCTCATCCACTATCAACAGAACCAGGCATCATG |
| CX3CR1 | NM_001 337.3 | 1040-1140 | GGGCGCTCAGTCCACGTTGATTTCTCCTCATCTGAATCACAAAGGAGCAGGCA TGGAAGTGTTCTGAGCAGCAATTTTACTTACCACACGAGTGATGGAG |
| CXCCR1 | NM_000 634.2 | 1950-2050 | GCAGCCACCAGTCCATTGGGCAGGCAGATGTTCCTAATAAAGCTTCTGTTCCG TGCTTGTCCCTGTGGAAGTATCTTGGTTGTGACAGAGTCAAGGGTGT |
| CXCL10 | NM_001 565.1 | 40-140 | GCAGAGGAACCTCCAGTCTCAGCACCATGAATCAAACTGCGATTCTGATTTGC TGCCTTATCTTTCTGACTCTAAGTGGCATTCAAGGAGTACCTCTCTC |
| CXCL12 | NM_199 168.2 | 505-605 | GGGCCTGAGGTTTGCCAGCATTTAGACCCTGCATTTATAGCATACGGTATGAT ATTGCAGCTTATATTCATCCATGCCCTGTACCTGTGCACGTTGGAAC |
| CXCL9 | NM_002 416.1 | 1975-2075 | CACCATCTCCCATGAAGAAAGGGAACGGTGAAGTACTAAGCGCTAGAGGAA GCAGCCAAGTCGGTTAGTGGAAGCATGATTGGTGCCCAGTTAGCCTCTG |
| CXCR3 | NM_001 504.1 | 80-180 | GTGAGTGACCACCAAGTGCTAAATGACGCCGAGGTTGCCGCCCTCCTGGAGA ACTTCAGCTCTTCCTATGACTATGGAGAAAACGAGAGTGACTCGTGCT |
| CXCR4 | NM_001 008540.1 | 135-235 | GTCACTATGGGAAAAGATGGGGAGGAGAGTTGTAGGATTCTACATTAATTCT CTTGTGCCCTTAGCCCACTACTTCAGAATTTCCTGAAGAAAGCAAGCC |
| CYORF14 | NR_001 544.2 | 143-243 | GAGGCTGTCTGCCAACATCTTTCATCACTCTGCCTGCAACTATGAAAAATTTA GTTCTAAAAAATGCAACCTTGCTAAATTGAGTACTAATAGGATTGGT |
| DAP10 | NM_001 007469.1 | 132-232 | ATCCTCTTCCTGCTTTTGCTCCCAGTGGCTGCAGCTCAGACGACTCCAGGAGA GAGATCATCACTCCCTGCCTTTTACCCTGGCACTTCAGGCTCTTGTT |
| DAP12 | NM_003 332.2 | 457-557 | CTGCACCTCATTCCAACTCCTACCGCGATACAGACCCACAGAGTGCCATCCCT GAGAGACCAGACCGCTCCCCAATACTCTCCTAAAATAAACATGAAGC |
| DEC1 | NM_017 418.2 | 190-290 | AGGCCTTACTTTCCAGATCCAGATCCTTGTGCATACAACTGACTTGTGTGGGT GAGGCTTGCAGAAAAAAATCAGCTAGAACAGCCTTGGGGGGTAGTGGCA |
| DNAM-1 | NM_006 566.2 | 163-263 | TAAACAGGATACGATAAAAGTCCTTAACCAAGACGCAGATGGGAAGAAGCG TTAGAGCGAGCAGCACTCACATCTCAAGAACCAGCCTTTCAAACAGTTT |

199

| DPP4 | NM_001 935.3 | 2700-2800 | CAGCAGTCAGCTCAGATCTCCAAAGCCCTGGTCGATGTTGGAGTGGATTTCCA GGCAATGTGGTATACTGATGAAGACCATGGAATAGCTAGCAGCACAG |
|---|---|---|---|
| EGLN1 | NM_022 051.1 | 3975-4075 | AGCAGCATGGACGACCTGATACGCCACTGTAACGGGAAGCTGGGCAGCTACA AAATCAATGGCCGGACGAAAGCCATGGT |
| EGLN3 | NM_022 073.3 | 800-900 | AAGCTACATGGTGGGATCCTGCGGATATTTCCAGAGGGGAAATCATTCATAG CAGATGTGGAGCCCATTTTTGACAGACTCCTGTTCTTCTGGTCAGATC |
| EIF1 | NM_005 801.3 | 869-969 | CCTGAACAGTCCTCGGTGAATCTGAGAGGAGAGGATGGGGTAAGGCAGAAG CACCAGCTGTACTACTAGAAGGGAGCTTTTGGTGGTAGATCCCCTGGTG |
| ELF4 | NM_001 421.3 | 335-435 | AGCTCTGGAGGGCTCTGATAATCCCGTTGTCAGCTCTCTGAAAAGACAGCATG GCTATTACCCTACAGCCCAGTGACCTGATCTTTGAGTTCGCAAGCAA |
| ENTPD1 | NM_001 776.4 | 225-325 | TTCGAGTAACTTTAGGAAAATGAGCTGCTGGACTCCTCAGTCAATCTGTCCTT TCTAGTCAATGAAAAAGACAGGGTTTGAGGTTCCTTCCGAAACGGGG |
| Eomes | NM_005 442.2 | 1670-1770 | ATCCCATGCCCTGGGGTATTACCCAGACCCAACCTTTCCTGCAATGGCAGGGT GGGGAGGTCGAGGTTCTTACCAGAGGAAGATGGCAGCTGGACTACCA |
| EPHA4 | NM_004 438.3 | 20-120 | GCAGCGTTGGCACCGGCGAACCATGGCTGGGATTTTCTATTTCGCCCTATTTT CGTGTCTCTTCGGGATTTGCGACGCTGTCACAGGTTCCAGGGTATAC |
| ETV6 | NM_001 987.4 | 3840-3940 | GTATGAATATGAAATCAGAGACCAGGGCATGATGTTGCTAGGATTAGAGCCT CTCAGTCTGGCCTCTTCACCCAAGTGCAAGAACTCAGTCTCTTACTGT |
| FADD | NM_003 824.2 | 1560-1660 | TGAGACTGCTAAGTAGGGGCAGTGATGGTTGCCAGGACGAATTGAGATAATA TCTGTGAGGTGCTGATGAGTGATTGACACACAGCACTCTCTAAATCTT |
| FANCC | NM_000 136.2 | 2130-2230 | GACTCAGTCAGACATGTTCACTAATGACTCAAGTGAGCCTTCGGTACTCCTGG TGCCCGCCCGGCCAGACCGTCAGCTTGATAATTACTAAAGCAAAGGC |
| FAS | NM_000 043.3 | 90-190 | CACCGGGGCTTTTCGTGAGCTCGTCTCTGATCTCGCGCAAGAGTGACACACAG GTGTTCAAAGACGCTTCTGGGGAGTGAGGGAAGCGGTTTACGAGTGA |
| FASLG | NM_000 639.1 | 625-725 | TCCATGCCTCTGGAATGGGAAGACACCTATGGAATTGTCCTGCTTTCTGGAGT GAAGTATAAGAAGGGTGGCCTTGTGATCAATGAAACTGGGCTGTACT |
| FLT1 | NM_002 019.2 | 5615-5715 | TTCAACTGCTTTGAAACTTGCCTGGGGTCTGAGCATGATGGGAATAGGGAGA CAGGGTAGGAAAGGGCGCCTACTCTTCAGGGTCTAAAGATCAAGTGGG |
| FLT3LG | NM_001 459.2 | 927-1027 | CCTCCCCAGAATGGAGGCAACGCCAGAATCCAGCACCGGCCCCATTTACCCA ACTCTGTACAAAGCCCTTGTCCCCATGAAATTGTATATAAATCATCCT |
| FOS | NM_005 252.2 | 1475-1575 | ACTCAAGTCCTTACCTCTTCCGGAGATGTAGCAAAACGCATGGAGTGTGTATT GTTCCCAGTGACACTTCAGAGAGCTGGTAGTTAGTAGCATGTTGAGC |
| FOXP3 | NM_014 009.3 | 1230-1330 | GGGCCATCCTGGAGGCTCCAGAGAAGCAGCGGACACTCAATGAGATCTACCA CTGGTTCACACGCATGTTTGCCTTCTTCAGAAACCATCCTGCCACCTG |
| FYN | NM_002 037.3 | 765-865 | GTCTTTGGAGGTGTGAACTCTTCGTCTCATACGGGGACCTTGCGTACGAGAGG AGGAACAGGAGTGACACTCTTTGTGGCCCTTTATGACTATGAAGCAC |
| FZD1 | NM_003 505.1 | 2430-2530 | GTGCCAATCCTGACATCTCGAGGTTTCCTCACTAGACAACTCTCTTTCGCAGG CTCCTTTGAACAACTCAGCTCCTGCAAAAGCTTCCGTCCCTGAGGCA |
| GAL3ST4 | NM_024 637.4 | 1140-1240 | CGAGCCCAAACCCTCAATCCCAATGCCCTCATCCATCCTGTTTCCACTGTTAC TGATCATCGCAGCCAGATATCAAGCCCTGCCTCTTTCGATTTGGGGT |
| GARNL4 | NM_015 085.4 | 4140-4240 | CCCACGGCTGGAAAGAGGCCTGTACGTTCTGGACGCGTTTTGTTGGCTGGGCT TCTGGAGGCACTGGCAAGGTCAAACTGCATTTCTTTAAGAACAGTTG |
| GAS2 | NM_005 256.3 | 915-1015 | GATCTCCCGTGTGGATGGCAAAACATCCCCTATCCAAAGCAAATCTCCAACTC TAAAGGACATGAATCCAGATAACTACTTGGTGGTCTCTGCCAGTTAT |
| GATA2 | NM_032 638.3 | 1495-1595 | GAAGAAGGAAGGGATCCAGACTCGGAACCGGAAGATGTCCAACAAGTCCAA GAAGAGCAAGAAAGGGGCGGAGTGCTTCGAGGAGCTGTCAAAGTGCATG |

| Gene | Accession | Position | Sequence |
|---|---|---|---|
| GATA3 | NM_001002295.1 | 2835-2935 | AAGAGTCCGGCGGCATCTGTCTTGTCCCTATTCCTGCAGCCTGTGCTGAGGGT AGCAGTGTATGAGCTACCAGCGTGCATGTCAGCGACCCTGGCCCGAC |
| Gfi1 | NM_005263.2 | 2235-2335 | TCATCACTGGAGGTAAAAGCACAAGCAATGCCTGTGGACAAGATGTCATTCA TTCACTCAGCAAATGTTCATGGATCACCGGCTACCAAGGTACCAGGCA |
| GILZ | NM_198057.2 | 1400-1500 | TTAAGCAGAGGCAACCTCTCTCTTCTCCTCTGTTTCGTGAAGGCAGGGGACAC AGATGGGAGAGATTGAGCCAAGTCAGCCTTCTGTTGGTTAATATGGT |
| GLIPR1 | NM_006851.2 | 255-355 | CTGCGTTCGAATCCATAACAAGTTCCGATCAGAGGTGAAACCAACAGCCAGT GATATGCTATACATGACTTGGGACCCAGCACTAGCCCAAATTGCAAAA |
| GLO1 | NM_006708.1 | 1240-1340 | GGAAATGATATGGTACCCAGACACTGGGCTAGGCTGCAACTTTATCTCATTTA ATACTCCCAGCTGTCATGTGAGAAAGAAAGCAGGCTAGGCATGTGAA |
| GSK3B | NM_002093.2 | 925-1025 | ACTGATTATACCTCTAGTATAGATGTATGGTCTGCTGGCTGTGTGTTGGCTGA GCTGTTACTAGGACAACCAATATTTCCAGGGGATAGTGGTGTGGATC |
| GZMA | NM_006144.2 | 155-255 | AGACCCTACATGGTCCTACTTAGTCTTGACAGAAAAACCATCTGTGCTGGGGC TTTGATTGCAAAAGACTGGGTGTTGACTGCAGCTCACTGTAACTTGA |
| GZMB | NM_004131.3 | 540-640 | ACACTACAAGAGGTGAAGATGACAGTGCAGGAAGATCGAAAGTGCGAATCT GACTTACGCCATTATTACGACAGTACCATTGAGTTGTGCGTGGGGGACC |
| GzmH | NM_033423.3 | 705-805 | AAAAAAGGGACACCTCCAGGAGTCTACATCAAGGTCTCACACTTCCTGCCCT GGATAAAGAGAACAATGAAGCGCCTCTAACAGCAGGCATGAGACTAAC |
| HDAC1 | NM_004964.2 | 785-885 | CAAGCCGGTCATGTCCAAAGTAATGGAGATGTTCCAGCCTAGTGCGGTGGTC TTACAGTGTGGCTCAGACTCCCTATCTGGGGATCGGTTAGGTTGCTTC |
| HDAC2 | NM_001527.1 | 930-1030 | AAGCCTATTATCTCAAAGGTGATGGAGATGTATCAACCTAGTGCTGTGGTATT ACAGTGTGGTGCAGACTCATTATCTGGTGATAGACTGGGTTGTTTCA |
| HES1 | NM_004649.5 | 1340-1440 | TTGAGTTAATCAGCGTAAGGGGATTTCTAAAGCAGGCAATCCCTGTAGCCGC AGAGAATAAACGCCTTCCCAAAATGGCAACTTCCCACAGCCACATTTC |
| HLA-A | NM_002116.5 | 1000-1100 | GGAAGAGCTCAGATAGAAAAGGAGGGAGTTACACTCAGGCTGCAAGCAGTG ACAGTGCCCAGGGCTCTGATGTGTCCCTCACAGCTTGTAAAGTGTGAGA |
| HOXA10 | NM_018951.3 | 1503-1603 | TTCTATAGAGATAGATATTGTCCTAAGTGTCAAGTCCTGACTGGGCTGGGTTT GCTGTCTTGGGGTCCCACTGCTCGAAATGGCCCCTGTCTTCGGCCGA |
| HOXA9 | NM_152739.3 | 1015-1115 | GGCTCTAAACCTCAGGCCACATCTTTTCCAAGGCAAACCCTGTTCAGGCTGGC TCGTAGGCCTGCCGCTTTGATGGAGGAGGTATTGTAAGCTTTCCATT |
| HOXB3 | NM_002146.4 | 60-160 | TGTCCGTTTAAATGCTGCTGGGAGACTCGTAAAAAAATCATCGTGGACCTGG AGGATGAGAGGGGCGAGCTTTATTTCGGTCGGATTGCGGTGTGGTGGT |
| HOXB4 | NM_024015.4 | 1340-1440 | CCTTTCTTTGTCCCCCACTCCCGATACCCAGCGAAAGCACCCTCTGACTGCCA GATAGTGCAGTGTTTTGGTCACGGTAACACACACACTCTCCCTCA |
| HPRT1 | NM_000194.1 | 240-340 | TGTGATGAAGGAGATGGGAGGCCATCACATTGTAGCCCTCTGTGTGCTCAAG GGGGGCTATAAATTCTTTGCTGACCTGCTGGATTACATCAAAGCACTG |
| HRH1 | NM_000861.2 | 3055-3155 | GTGGCAGCTCAAAATGATATGTTTGAGTAGACGAACAGCTGACATGGAGTTC CCGTGCACCTACGGAAGGGGACGCTTTGAAGGAACCAAGTGCATTTTT |
| HRH2 | NM_022304.1 | 600-700 | GCGGTCCTCATCCTCATCACCGTTGCTGGCAATGTGGTCGTCTGTCTGGCCGT GGGCTTGAACCGCCGGCTCCGCAACCTGACCAATTGTTTCATCGTGT |
| IAP | NM_001777.3 | 897-997 | GCCATATTGGTTATTCAGGTGATAGCCTATATCCTCGCTGTGGTTGGACTGAG TCTCTGTATTGCGGCGTGTATACCAATGCATGGCCCTCTTCTGATTT |
| ICOS | NM_012092.2 | 640-740 | AACTCTGGCACCCAGGCATGAAGCACGTTGGCCAGTTTTCCTCAACTTGAAGT GCAAGATTCTCTTATTTCCGGGACCACGGAGAGTCTGACTTAACTAC |
| ICOSLG | NM_015259.4 | 1190-1290 | CTGCTGGCGTTGGCTGTGATCCTGGAATGAGGCCCTTTCAAAAGCGTCATCCA CACCAAAGGCAAATGTCCCCAAGTGAGTGGGCTCCCCGCTGTCACTG |

| Gene | Accession | Position | Sequence |
|---|---|---|---|
| ID2 | NM_002166.4 | 505-605 | CGGATATCAGCATCCTGTCCTTGCAGGCTTCTGAATTCCCTTCTGAGTTAATGTCAAATGACAGCAAAGCACTGTGTGGCTGAATAAGCGGTGTTCATGA |
| IFNa1 | NM_024013.1 | 585-685 | ATCCCTCTCTTTATCAACAAACTTGCAAGAAAGATTAAGGAGGAAGGAATAACATCTGGTCCAACATGAAAACAATTCTTATTGACTCATACACCAGGTC |
| IFNG | NM_000619.2 | 970-1070 | ATACTATCCAGTTACTGCCGGTTTGAAAATATGCCTGCAATCTGAGCCAGTGCTTTAATGGCATGTCAGACAGAACTTGAATGTGTCAGGTGACCCTGAT |
| IFNGR1 | NM_000416.1 | 1140-1240 | CCCGGGCAGCCATCTGACTCCAATAGAGAGAGAGAGTTCTTCACCTTTAAGTAGTAACCAGTCTGAACCTGGCAGCATCGCTTTAAACTCGTATCACTCC |
| IGF1R | NM_000875.2 | 455-555 | TCGGGGGGCCATCAGGATTGAGAAAAATGCTGACCTCTGTTACCTCTCCACTGTGGACTGGTCCCTGATCCTGGATGCGGTGTCCAATAACTACATTGTG |
| IKZF1 | NM_006060.3 | 4485-4585 | CCGCTGTGTACTACTGTGTGCCTAGATTCCATGCACTCTCGTTGTGTTTGAAGTAAATATTGGAGACCGGAGGGTAACAGGTTGGCCTGTTGATTACAGC |
| IL10 | NM_000572.2 | 230-330 | AAGGATCAGCTGGACAACTTGTTGTTAAAGGAGTCCTTGCTGGAGGACTTTAAGGGTTACCTGGGTTGCCAAGCCTTGTCTGAGATGATCCAGTTTTACC |
| IL10RA | NM_001558.2 | 150-250 | TGCCCAGCCCTCCGTCTGTGTGGTTTGAAGCAGAATTTTTCCACCACATCCTCCACTGGACACCCATCCCAAATCAGTCTGAAAGTACCTGCTATGAAGT |
| IL12A | NM_000882.2 | 775-875 | CTTTCTAGATCAAAACATGCTGGCAGTTATTGATGAGCTGATGCAGGCCCTGAATTTCAACAGTGAGACTGTGCCACAAAAATCCTCCCTTGAAGAACCG |
| IL12RB1 | NM_005535.1 | 1292-1392 | AGGAAAAGTGTTACTACATTACCATCTTTGCCTCTGCGCACCCCGAGAAGCTCACCTTGTGGTCTACGGTCCTGTCCACCTACCACTTTGGGGGCAATGC |
| IL12RB2 | NM_001559.2 | 1315-1415 | CCTCCGTGGGACATTAGAATCAAATTTCAAAAGGCTTCTGTGAGCAGATGTACCCTTTATTGGAGAGATGAGGGACTGGTACTGCTTAATCGACTCAGAT |
| IL13 | NM_002188.2 | 516-616 | TTTCTTTCTGATGTCAAAAATGTCTTGGGTAGGCGGGAAGGAGGGTTAGGGAGGGTAAAATTCCTTAGCTTAGACCTCAGCCTGTGCTGCCCGTCTTCA |
| IL15 | NM_172174.1 | 1685-1785 | AGGGTGATAGTCAAATTATGTATTGGTGGGGCTGGGTACCAATGCTGCAGGTCAACAGCTATGCTGGTAGGCTCCTGCCAGTGTGGAACCACTGACTACT |
| IL15Ra | NM_002189.2 | 39-139 | CGCTCGCCCGGGGAGTCCAGCGGTGTCCTGTGGAGCTGCCGCCATGGCCCCGCGGCGGGCGCGCGGCTGCCGGACCCTCGGTCTCCCGGCGCTGCTACTG |
| IL17A | NM_002190.2 | 240-340 | TACTACAACCGATCCACCTCACCTTGGAATCTCCACCGCAATGAGGACCCTGAGAGATATCCCTCTGTGATCTGGGAGGCAAAGTGCCGCCACTTGGGCT |
| IL17F | NM_052872.3 | 210-310 | GCCCGCCTGTGCCAGGAGGTAGTATGAAGCTTGACATTGGCATCATCAATGAAAACCAGCGCGTTTCCATGTCACGTAACATCGAGAGCCGCTCCACCTC |
| IL17RA | NM_014339.4 | 3020-3120 | CTACTATGTGGCGGGCATTTGGGATACCAAGATAAATTGCATGCGGCATGGCCCCAGCCATGAAGGAACTTAACCGCTAGTGCCGAGGACACGTTAAACG |
| IL18 | NM_001562.2 | 48-148 | GACAGTCAGCAAGGAATTGTCTCCCAGTGCATTTTGCCCTCCTGGCTGCCAACTCTGGCTGCTAAAGCGGCTGCCACCTGCTGCAGTCTACACAGCTTCG |
| IL18R1 | NM_003855.2 | 2025-2125 | GAATGAGGGGATTTTAAGTGTCTGAAGAGGCATTTTCTAGGGACCAGTGGGTGACTGAGTAACTGAAATGCTGCTTTCACTCCCTAACACCATGGATCTG |
| IL18RAP | NM_003853.2 | 2412-2512 | GCTTGATGGACAATGGAGTGGGATTGAGACTGTGGTTTAGAGCCTTTGATTTCCTGGACTGGACTGACGGCGAGTGAATTCTCTAGACCTTGGGTACTTT |
| IL2 | NM_000586.2 | 300-400 | AGGATGCAACTCCTGTCTTGCATTGCACTAAGTCTTGCACTTGTCACAAACAGTGCACCTACTTCAAGTTCTACAAAGAAAACACAGCTACAACTGGAGC |
| IL21R | NM_021798.2 | 2080-2180 | CGTGTTTGTGGTCAACAGATGACAACAGCCGTCCTCCCTCCTAGGGTCTTGTGTTGCAAGTTGGTCCACAGCATCTCCGGGGCTTTGTGGGATCAGGGCA |
| IL22 | NM_020525.4 | 319-419 | CTATCTGATGAAGCAGGTGCTGAACTTCACCCTTGAAGAAGTGCTGTTCCCTCAATCTGATAGGTTCCAGCCTTATATGCAGGAGGTGGTGCCCTTCCTG |

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|---|---|---|---|
| IL23R | NM_144701.2 | 710-810 | AACTGCAAATTCACCTGGATGATATAGTGATACCTTCTGCAGCCGTCATTTCC AGGGCTGAGACTATAAATGCTACAGTGCCCAAGACCATAATTTATTG |
| IL2RA | NM_000417.1 | 1000-1100 | CTTGGTAAGAAGCCGGGAACAGACAACAGAAGTCATGAAGCCCAAGTGAAA TCAAAGGTGCTAAATGGTCGCCCAGGAGACATCCGTTGTGCTTGCCTGC |
| IL2RB | NM_000878.2 | 1980-2080 | GTCCTGCTGCCCGAGCCAGGAACTGTGTGTGTTGCAGGGGGGCAGTAACTCC CCAACTCCCTCGTTAATCACAGGATCCCACGAATTTAGGCTCAGAAGC |
| IL2RG | NM_000206.1 | 595-695 | CCACAGCTGGACTGAACAATCAGTGGATTATAGACATAAGTTCTCCTTGCCTA GTGTGGATGGGCAGAAACGCTACACGTTTCGTGTTCGGAGCCGCTTT |
| IL4 | NM_000589.2 | 625-725 | GACACTCGCTGCCTGGGTGCGACTGCACAGCAGTTCCACAGGCACAAGCAGC TGATCCGATTCCTGAAACGGCTCGACAGGAACCTCTGGGGCCTGGCGG |
| IL4R | NM_000418.2 | 705-805 | ATCATCTCACCTATGCAGTCAACATTTGGAGTGAAAACGACCCGGCAGATTTC AGAATCTATAACGTGACCTACCTAGAACCCTCCCTCCGCATCGCAGC |
| IL5 | NM_000879.2 | 105-205 | CCACAGAAATTCCCACAAGTGCATTGGTGAAAGAGACCTTGGCACTGCTTTCT ACTCATCGAACTCTGCTGATAGCCAATGAGACTCTGAGGATTCCTGT |
| IL6 | NM_000600.1 | 220-320 | TGACAAACAAATTCGGTACATCCTCGACGGCATCTCAGCCCTGAGAAAGGAG ACATGTAACAAGAGTAACATGTGTGAAAGCAGCAAAGAGGCACTGGCA |
| IL6R | NM_000565.2 | 993-1093 | CTTTCTACATAGTGTCCATGTGCGTCGCCAGTAGTGTCGGGAGCAAGTTCAGC AAAACTCAAACCTTTCAGGGTTGTGGAATCTTGCAGCCTGATCCGCC |
| IL7R | NM_002185.2 | 1610-1710 | TTGCTTTGACCACTCTTCCTGAGTTCAGTGGCACTCAACATGAGTCAAGAGCA TCCTGCTTCTACCATGTGGATTTGGTCACAAGGTTTAAGGTGACCCA |
| IL9 | NM_000590.1 | 300-400 | AAGTACTAAAGAACAACAAGTGTCCATATTTTTCCTGTGAACAGCCATGCAA CCAAACCACGGCAGGCAACGCGCTGACATTTCTGAAGAGTCTTCTGGA |
| INDO | NM_002164.3 | 50-150 | CTATTATAAGATGCTCTGAAAAACTCTTCAGACACTGAGGGGCACCAGAGGAG CAGACTACAAGAATGGCACACGCTATGGAAAACTCCTGGACAATCAGT |
| IRF1 | NM_002198.1 | 510-610 | CTGTGCGAGTGTACCGGATGCTTCCACCTCTCACCAAGAACCAGAGAAAAGA AAGAAAGTCGAAGTCCAGCCGAGATGCTAAGAGCAAGGCCAAGAGGAA |
| IRF2 | NM_002199.2 | 1375-1475 | CAGTACCTGGAGCTTCTCTTTAACTCAGGACTCCAGCCCATTGGTAGACGTGT GTTTCTAGAGCCTGCTGGATCTCCCAGGGCTACTCACTCAAGTTCAA |
| IRF4 | NM_002460.1 | 325-425 | GGGCACTGTTTAAAGGAAAGTTCCGAGAAGGCATCGACAAGCCGGACCCTCC CACCTGGAAGACGCGCCTGCGGTGCGCTTTGAACAAGAGCAATGACTT |
| ITGA1 | NM_181501.1 | 1875-1975 | AAGTGGCAAGACTATAAGGAAAGAGTATGCACAACGTATTCCATCAGGTGGG GATGGTAAGACACTGAAATTTTTTGGCCAGTCTATCCACGGAGAAATG |
| ITGA4 | NM_000885.4 | 975-1075 | GCCCACTGCCAACTGGCTCGCCAACGCTTCAGTGATCAATCCCGGGGCGATTT ACAGATGCAGGATCGGAAAGAATCCCGGCCAGACGTGCGAACAGCTC |
| ITGA5 | NM_002205.2 | 925-1025 | AGAAGACTTTGTTGCTGGTGTGCCCAAAGGGAACCTCACTTACGGCTATGTCA CCATCCTTAATGGCTCAGACATTCGATCCCTCTACAACTTCTCAGGG |
| ITGAL | NM_002209.2 | 3905-4005 | GTGAGGGCTTGTCATTACCAGACGGTTCACCAGCCTCTCTTGGTTTCCTTCCTT GGAAGAGAATGTCTGATCTAAATGTGGAGAAACTGTAGTCTCAGGA |
| ITGB1 | NM_033666.2 | 2000-2100 | TTTTAACATTACCAAGGTAGAAAGTCGGGACAAATTACCCCAGCCGGTCCAA CCTGATCCTGTGTCCCATTGTAAGGAGAAGGATGTTGACGACTGTTGG |
| ITK | NM_005546.3 | 3430-3530 | GCCAGTAAAGAAGTCAGTATAGAACCACTAGCGAATAGTGTTGCTCTGGCAC AGACCACTGTGGTTGATGGCATGGCCCTCCAACTTGGAATAGGATTTT |
| JAK1 | NM_002227.1 | 285-385 | GAGAACACCAAGCTCTGGTATGCTCCAAATCGCACCATCACCGTTGATGACA AGATGTCCCTCCGGCTCCACTACCGGATGAGGTTCTATTTCACCAATT |
| JAK2 | NM_004972.2 | 455-555 | CTCCTCCCGCGACGGCAAATGTTCTGAAAAAGACTCTGCATGGGAATGGCCT GCCTTACGATGACAGAAATGGAGGGAACATCCACCTCTTCTATATATC |

203

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|---|---|---|---|
| JAK3 | NM_000 215.2 | 1715-1815 | GTGCTGCTGAAGGTCATGGATGCCAAGCACAAGAACTGCATGGAGTCATTCC TGGAAGCAGCGAGCTTGATGAGCCAAGTGTCGTACCGGCATCTCGTGC |
| JunB | NM_002 229.2 | 1155-1255 | GCGCGCCTGGAGGACAAGGTGAAGACGCTCAAGGCCGAGAACGCGGGGCTG TCGAGTACCGCCGGCCTCCTCCGGGAGCAGGTGGCCCAGCTCAAACAGA |
| KIR2DL1 (NKAT1)/C D158a | NM_014 218.2 | 881-981 | GCAGGAAACAGAACAGCGAATAGCGAGGACTCTGATGAACAAGACCCTCAG GAGGTGACATACACACAGTTGAATCACTGCGTTTTCACACAGAGAAAAA |
| KIR2DL2 (NKAT6)/C D158b | NM_014 219.2 | 814-914 | TCTCCTTCATCGCTGGTGCTCCAACAAAAAAAATGCTGCGGTAATGGACCAA GAGTCTGCAGGGAACAGAACAGCGAATAGCGAGGACTCTGATGAACAA |
| KIR2DL3 (NKAT2)/C D158b | NM_015 868.2 | 741-841 | CTCCGAAACCGGTAACCCCAGACACCTGCATGTTCTGATTGGGACCTCAGTGG TCATCATCCTCTTCATCCTCCTCCTCTTCTTTCTCCTTCATCGCTGG |
| KIR2DL4 (p 49 CD158d) | NM_002 255.5 | 15-115 | GCGTCCTGGCAGCAGAAGCTGCACCATGTCCATGTCACCCACGGTCATCATCC TGGCATGTCTTGGGTTCTTCTTGGACCAGAGTGTGTGGGCACACGTG |
| KIR2DL5A | NM_020 535.3 | 1451-1551 | GACACGTGCTGTTCCACCTTCCCTCATGCTGTTTCACCTTTCCTCAGACTATTT TCCAGCCTTCTGTCAGTCAGCAGTGAAACTTATAAAATTTTTTGTG |
| KIR2DS1 | NM_014 512.1 | 698-798 | CTTCACCCACTGAACCAAGCTCCGAAACCGGTAACCCCAGACACCTACATGT TCTGATTGGGACCTCAGTGGTCAAAATCCCTTTCACCATCCTCCTCTT |
| KIR2DS2 (NKAT5)/C D158b | NM_012 312.2 | 856-956 | CAAGAGCCTGCAGGGAACAGAACAGTGAACAGCGAGGATTCTGATGAACAA GACCATCAGGAGGTGTCATACGCATAATTGGATCACTGTGTTTTCACAC |
| KIR2DS3 (NKAT7) | NM_012 313.1 | 693-793 | GGCCTTCACCCACTGAACCAAGCTCCAAAACCGGTAACCCCAGACACCTACA CGTTCTGATTGGGACCTCAGTGGTCAAACTCCCTTTCACCATCCTCCT |
| KIR2DS4 (NKAT8) | NM_012 314.3 | 1427-1527 | ACATACAAGAGGCTGCCTCTTAACACAGCACTTAGACACGTGCTGTTCCACCT CCCTTCAGACTATCTTTCAGCCTTCTGCCAGCAGTAAAACTTATAAA |
| KIR2DS5 (NKAT9) | NM_014 513.2 | 204-304 | CTTCCTTCTGCACAGAGAGGGGACGTTTAACCACACTTTGCGCCTCATTGGAG AGCACATTGATGGGGTCTCCAAGGGCAACTTCTCCATCGGTCGCATG |
| KIR3DL1 (NKAT3/NK B1) | NM_013 289.2 | 1054-1154 | CCAAATCTGGTAACCCCAGACACCTGCACATTCTGATTGGGACCTCAGTGGTC ATCATCCTCTTCATCCTCCTCCTCTTCTTTCTCCTTCATCTCTGGTG |
| KIR3DL2 ( NKAT4) | NM_006 737.2 | 884-984 | TGCCACCCACGGAGGGACCTACAGATGCTTCGGCTCTTTCCGTGCCCTGCCCT GCGTGTGGTCAAACTCAAGTGACCCACTGCTTGTTTCTGTCACAGGA |
| KIR3DL3 (KIRC1 CD158z) | NM_153 443.3 | 508-608 | CCTTGCGCCTCGTTGGACAGCTCCACGATGCGGGTTCCCAGGTCAACTATTCC ATGGGTCCCATGACACCTGCCCTTGCAGGGACCTACAGATGCTTTGG |
| KIR3DS1 ( NKAT10) | NM_001 083539.1 | 1000-1100 | CTCCAAATCTGGTAACCTCAGACACCTGCACATTCTGATTGGGACCTCAGTGG TCAAAATCCCTTTCACCATCCTCCTCTTCTTTCTCCTTCATCGCTGG |
| KIT | NM_000 222.1 | 5-105 | CATCGCAGCTACCGCGATGAGAGGCGCTCGCGGCGCCTGGGATTTTCTCTGCG TTCTGCTCCTACTGCTTCGCGTCCAGACAGGCTCTTCTCAACCATCT |
| KLF10 | NM_005 655.1 | 570-670 | GCTCAGGCAACAAGTGTGATTCGTCATACAGCTGATGCCCAGCTATGTAACC ACCAGACCTGCCCAATGAAAGCAGCCAGCATCCTCAACTATCAGAACA |
| Klf2 | NM_016 270.2 | 1015-1115 | GGAAGTTTGCGCGCTCAGACGAGCTCACGCGCCACTACCGAAAGCACACGGG CCACCGGCCATTCCAGTGCCATCTGTGCGATCGTGCCTTCTCGCGCTC |
| KLF4 | NM_004 235.4 | 1980-2080 | CGAGCATTTTCCAGGTCGGACCACCTCGCCTTACACATGAAGAGGCATTTTTA AATCCCAGACAGTGGATATGACCCACACTGCCAGAAGAGAATTCAGT |

| Gene | Accession | Position | Sequence |
|------|-----------|----------|----------|
| KLF6 | NM_001 008490.1 | 1165-1265 | GGGATGCGTGTTCCAGCCAAAGCATGCCGTTCTGCACCCTACCCAGTTGCCTCCAGGGCCTCTCCTTGGAAGGTCTTTTGAGGGCTAAAAAGGTCCTGTA |
| KLRB1 | NM_002 258.2 | 85-185 | TGAGTTAAACTTACCCACAGACTCAGGCCCAGAAAGTTCTTCACCTTCATCTCTTCCTCGGGATGTCTGTCAGGGTTCACCTTGGCATCAATTTGCCCTG |
| KLRC1 | NM_002 259.3 | 335-435 | ACCTATCACTGCAAAGATTTACCATCAGCTCCAGAGAAGCTCATTGTTGGGATCCTGGGAATTATCTGTCTTATCTTAATGGCCTCTGTGGTAACGATAG |
| KLRD1 (CD94) | NM_002 262.3 | 542-642 | AGCCTGCTTCAGCTTCAAAACACAGATGAACTGGATTTTATGAGCTCCAGTCAACAATTTTACTGGATTGGACTCTCTTACAGTGAGGAGCACACCGCCT |
| KLRG1 | NM_005 810.3 | 45-145 | TGCCTACGGCAACCCAAGCCCAGAATGACTATGGACCACAGCAAAAATCTTCCTCTTCCAGGCCTTCTTGTTCTTGCCTTGTGGCAATAGCTTTGGGGCT |
| LAIR1 | NM_002 287.3 | 1195-1295 | GCACCTGAGGGTAGAAAGTCACTCTAGGAAAAGCCTGAAGCAGCCATTTGGAAGGCTTCCTGTTGGATTCCTCTTCATCTAGAAAGCCAGCCAGGCAGCT |
| LCK | NM_005 356.2 | 1260-1360 | ATTAAGTGGACAGCGCCAGAAGCCATTAACTACGGGACATTCACCATCAAGTCAGATGTGTGGTCTTTTGGGATCCTGCTGACGGAAATTGTCACCCACG |
| LDHA | NM_005 566.1 | 985-1085 | CAGAATGGAATCTCAGACCTTGTGAAGGTGACTCTGACTTCTGAGGAAGAGGCCCGTTTGAAGAAGAGTGCAGATACACTTTGGGGGATCCAAAAGGAGC |
| Lef1 | NM_016 269.3 | 1165-1265 | CCGTCACACATCCCATCAGATGTCAACTCCAAACAAGGCATGTCCAGACATCCTCCAGCTCCTGATATCCCTACTTTTTATCCCTTGTCTCCGGGTGGTG |
| LGALS3 | NM_002 306.2 | 120-220 | CAGCCGTCCGGAGCCAGCCAACGAGCGGAAAATGGCAGACAATTTTTCGCTCCATGATGCGTTATCTGGGTCTGGAAACCCAAACCCTCAAGGATGGCCT |
| LNK | NM_005 475.2 | 4285-4385 | CCTCCAGCCAGAAGTTAAACATCTGGGATATGACGTCTTCATGCCAGGGGCACTCATTTCTTAGCAGCCTCTCTACATACATCTCTCAGGTGGTGCCAAG |
| LOC282997 | NR_026 932.1 | 665-765 | TGATCACATTCTACCTGGCATTATTTCATCTGAGTCCCTGTCCTAGCCCTTCTGCCCATTAGACTGTAACCTTGTTTAGGGAAAGACCTGTGTCTTACTC |
| LRP5 | NM_002 335.1 | 2515-2615 | TGGACACCAACATGATCGAGTCGTCCAACATGCTGGGTCAGGAGCGGGTCGTGATTGCCGACGATCTCCCGCACCCGTTCGGTCTGACGCAGTACAGCGA |
| LRP6 | NM_002 336.1 | 2185-2285 | CTTAGATTATCCAGAAGGCATGGCAGTAGACTGGCTTGGGAAGAACTTGTACTGGGCAGACACAGGAACGAATCGAATTGAGGTGTCAAAGTTGGATGGG |
| LRRC32 | NM_005 512.2 | 3470-3570 | CACCCTGGTGTGGGTTCTCCTGTTCTCTCTGTGCTCTTGCATTCTCTCATTCCCTTTTCCTCTATTGAGCAGAGCCTGGAGTTTGAGACTATGGAATCCA |
| MAD1L1 | NM_003 550.2 | 306-406 | GAAGACCTGGGGGAAAACACCATGGTTTTATCCACCCTGAGATCTTTGAACAACTTCATCTCTCAGCGTGTGGAGGGAGGCTCTGGACTGGATATTTCTA |
| MAP2K1 | NM_002 755.2 | 970-1070 | ACGGAATGGACAGCCGACCTCCCATGGCAATTTTTGAGTTGTTGGATTACATAGTCAACGAGCCTCCTCCAAAACTGCCCAGTGGAGTGTTCAGTCTGGA |
| MAPK14 | NM_001 315.1 | 450-550 | TGGGCTCTGGCGCCTATGGCTCTGTGTGTGCTGCTTTTGACACAAAAACGGGGTTACGTGTGGCAGTGAAGAAGCTCTCCAGACCATTTCAGTCCATCAT |
| MAPK3 | NM_002 746.2 | 580-680 | AACGTGCTCCACCGAGATCTAAAGCCCTCCAACCTGCTCATCAACACCACCTGCGACCTTAAGATTTGTGATTTCGGCCTGGCCCGGATTGCCGATCCTG |
| MAPK8 | NM_139 049.1 | 945-1045 | TCTCTGTAGATGAAGCTCTCCAACACCCGTACATCAATGTCTGGTATGATCCTTCTGAAGCAGAAGCTCCACCACCAAAGATCCCTGACAAGCAGTTAGA |
| MCL1 | NM_021 960.3 | 1260-1360 | GCTGTAACTTCCTAGAGTTGCACCCTAGCAACCTAGCCAGAAAAGCAAGTGGCAAGAGGATTATGGCTAACAAGAATAAATACATGGGAAGAGTGCTCCC |
| MIF | NM_002 415.1 | 319-419 | TCCTACAGCAAGCTGCTGTGCGGCCTGCTGGCCGAGCGCCTGCGCATCAGCCCGGACAGGGTCTACATCAACTATTACGACATGAACGCGGCCAATGTGG |
| MMP14 | NM_004 995.2 | 1470-1570 | GACAAGATTGATGCTGCTCTCTTCTGGATGCCCAATGGAAAGACCTACTTCTTCCGTGGAAACAAGTACTACCGTTTCAACGAAGAGCTCAGGGCAGTGG |

205

| MPL | NM_005 373.2 | 895-995 | CAGTGGCACTTGGACTGCAATGCTTTACCTTGGACCTGAAGAATGTTACCTGT CAATGGCAGCAACAGGACCATGCTAGCTCCCAAGGCTTCTTCTACCA |
|---|---|---|---|
| MYB | NM_005 375.2 | 3145-3245 | AACTGTTGCATGGATCCTGTGTTTGCAACTGGGGAGACAGAAACTGTGGTTG ATAGCCAGTCACTGCCTTAAGAACATTTGATGCAAGATGGCCAGCACT |
| Myc | NM_002 467.3 | 1610-1710 | TCGGACACCGAGGAGAATGTCAAGAGGCGAACACACAACGTCTTGGAGCGCC AGAGGAGGAACGAGCTAAAACGGAGCTTTTTTGCCCTGCGTGACCAGA |
| MYO6 | NM_004 999.3 | 6655-6755 | AAGTTGGGGAGATGGCACCTTCTCAGAGGATTGTGAAAATATGAGGAAGAAA CAAAACAGTGCATGTAGGAGCACAGGGCCACACAAAGGCATTCTATTG |
| NBEA | NM_015 678.3 | 8645-8745 | CTGAGAGCCCTTGAAGGACCAGAAAACTGCTTATTCCCACGCTTGATATCTGT CTCCAGCGAAGGCCACTGTATCATATACTATGAACGAGGGCGATTCA |
| NCAM1 | NM_000 615.5 | 1620-1720 | GGTATTTGCCTATCCCAGTGCCACGATCTCATGGTTTCGGGATGGCCAGCTGC TGCCAAGCTCCAATTACAGCAATATCAAGATCTACAACACCCCCTCT |
| NCL | NM_005 381.2 | 1492-1592 | GAACAGAGATCGATGGGCGATCTATTTCCCTGTACTATACTGGAGAGAAAGG TCAAAATCAAGACTATAGAGGTGGAAAGAATAGCACTTGGAGTGGTGA |
| NFAT5 | NM_173 214.1 | 3290-3390 | CCCTGACAACTATTCAAACCCAGGACATCTCACAGCCTGGTACTTTTCCAGCA GTTTCTGCTTCTAGTCAGCTGCCCAACAGCGATGCACTATTGCAGCA |
| NFATC1 | NM_172 390.1 | 2510-2610 | CCAGTACCAGCGTTTCACCTACCTTCCCGCCAACGGTAACGCCATCTTTCTAA CCGTAAGCCGTGAACATGAGCGCGTGGGGTGCTTTTTCTAAAGACGC |
| NFATC2 | NM_012 340.3 | 1815-1915 | GACGGACATTGGAAGAAAGAACACGCGGGTGAGACTGGTTTTCCGAGTTCAC ATCCCAGAGTCCAGTGGCAGAATCGTCTCTTTACAGACTGCATCTAAC |
| NFATC3 | NM_004 555.2 | 2190-2290 | GTCCTTGAAGTTCCTCCATATCATAACCCAGCAGTTACAGCTGCAGTGCAGGT GCACTTTTATCTTTGCAATGGCAAGAGGAAAAAAAGCCAGTCTCAAC |
| NKG2C | NM_002 260.3 | 942-1042 | TATGTGAGTCAGCTTATAGGAAGTACCAAGAACAGTCAAACCCATGGAGACA GAAAGTAGAATAGTGGTTGCCAATGTCTCAGGGAGGTTGAAATAGGAG |
| NKG2D | NM_007 360.1 | 760-860 | GGACCAGGATTTACTTAAACTGGTGAAGTCATATCATTGGATGGGACTAGTA CACATTCCAACAAATGGATCTTGGCAGTGGGAAGATGGCTCCATTCTC |
| NKG2E | NM_002 261.2 | 760-860 | ACTCCTGAGCTCAAGAAATCAACACATCTTGGCCTCCCAAGTTGCTGGGATTA CTGACACAAGCCACCGCCCCTGAGTGCTCATGTACCATTTAGCTTGT |
| NKG2F | NM_013 431.2 | 29-129 | TTATATTGGTCAACAGCAAAATGAACATTACTACTCAGCCTCCAACACATGCA GTTTGCCTATACCAGGGATCCTGTCAAAATATACACCACTTATAGCT |
| NKp30 (CD337) | NM_147 130.1 | 50-150 | GCATCTGTCCTCTCTCCTCAGGGAGGCAAGCATTTGATGCTCGAGGTCCCTGG CAGTTGTGGTCCTTGGCAAGTGATGTGTGAGTCCCGTGTGTCATAGG |
| NKp44 (CD336) | NM_004 828.3 | 798-898 | CTTCAACAGGTCACGGACCTTCCCTGGACCTCAGTTTCCTCACCTGTAGAGAG AGAAATATTATATCACACTGTTGCAAGGACTAAGATAAGCGATGATG |
| NKp46 (CD335) | NM_001 145457.1 | 145-245 | TTTCATGGTTCCAAAGGAAAAGCAAGTGACCATCTGTTGCCAGGGAAATTAT GGGGCTGTTGAATACCAGCTGCACTTTGAAGGAAGCCTTTTTGCCGTG |
| NKp80 | NM_016 523.1 | 275-375 | AAAAAGGAAGTTGTTCAAATGCCACTCAGTATGAGGACACTGGAGATCTAAA AGTGAATAATGGCACAAGAAGAAATATAAGTAATAAGGACCTTTGTGC |
| NOS2 | NM_000 625.4 | 605-705 | TTGCCTGGGGGTCCATTATGACTCCCAAAAGTTTGACCAGAGGACCCAGGGAC AAGCCTACCCCTCCAGATGAGCTTCTACCTCAAGCTATCGAATTTGTC |
| Notch1 | NM_017 617.3 | 735-835 | CTGCCAGGCTTCACCGGCCAGAACTGTGAGGAAAATATCGACGATTGTCCAG GAAACAACTGCAAGAACGGGGGTGCCTGTGTGGACGGCGTGAACACCT |
| NR3C1 | NM_001 018077.1 | 1665-1765 | GCTTTCTCCTCTGGCGGGAGAAGACGATTCATTCCTTTTGGAAGGAAACTCGA ATGAGGACTGCAAGCCTCTCATTTTACCGGACACTAAACCCAAAATT |
| NR4A1 | NM_002 135.3 | 155-255 | CGGCCGGGTAGGGTGCAGCCTGAGGCTTGTTCAGCAGAACAGGTGCAAGCCA CATTGTTGCCAAGACCTGCCTGAAGCCGGATTCTCCCCACTGCCTCCT |

206

| NRIP1 | NM_003<br>489.2 | 335-435 | TGACTCATGGAGAAGAGCTTGGCTCTGATGTGCACCAGGATTCTATTGTTTTA<br>ACTTACCTAGAAGGATTACTAATGCATCAGGCAGCAGGGGGATCAGG |
|---|---|---|---|
| NT5E | NM_002<br>526.2 | 1214-<br>1314 | ATTCGGGTTTTGAAATGGATAAACTCATCGCTCAGAAAGTGAGGGGTGTGGA<br>CGTCGTGGTGGGAGGACACTCCAACACATTTCTTTACACAGGCAATCC |
| OPTN | NM_001<br>008211.1 | 625-725 | TGAAGCTAAATAATCAAGCCATGAAAGGGAGATTTGAGGAGCTTTCGGCCTG<br>GACAGAGAAACAGAAGGAAGAACGCCAGTTTTTTGAGATACAGAGCAA |
| P2RX7 | NM_002<br>562.4 | 340-440 | AGTTGGTGCACAGTGTCTTTGACACCGCAGACTACACCTTCCCTTTGCAGGGG<br>AACTCTTTCTTCGTGATGACAAACTTTCTCAAAACAGAAGGCCAAGA |
| p38 | NM_006<br>303.3 | 507-607 | CCCTCTCCCTGCTTGTGCTGCACAGGCTGCTCTGTGAGCACTTCAGGGTCCTG<br>TCCACGGTGCACACGCACTCCTCGGTCAAGAGCGTGCCTGAAAACCT |
| Pax5 | NM_016<br>734.1 | 2288-<br>2388 | CTCCAAGAGGAGCACACTTTGGGGAGATGTCCTGGTTTCCTGCCTCCATTTCT<br>CTGGGACCGATGCAGTATCAGCAGCTCTTTTCCAGATCAAAGAACTC |
| PDCD1 | NM_005<br>018.1 | 175-275 | CTTCTTCCCAGCCCTGCTCGTGGTGACCGAAGGGGACAACGCCACCTTCACCT<br>GCAGCTTCTCCAACACATCGGAGAGCTTCGTGCTAAACTGGTACCGC |
| PDCD1LG2 | NM_025<br>239.3 | 235-335 | TGTGGAGCTGTGGCAAGTCCTCATATCAAATACAGAACATGATCTTCCTCCTG<br>CTAATGTTGAGCCTGGAATTGCAGCTTCACCAGATAGCAGCTTTATT |
| PDE3 | NM_000<br>921.3 | 3010-<br>3110 | CTGGCCAACCTTCAGGAATCCTTCATCTCTCACATTGTGGGGCCTCTGTGCAA<br>CTCCTATGATTCAGCAGGACTAATGCCTGGAAAATGGGTGGAAGACA |
| PDE4 | NM_001<br>111307.1 | 3855-<br>3955 | AATAATGGTGTATACCCTCATTCTCATTCCTGGGCAGCCCTTCCTTCCACCCTG<br>GCACCAAAATAATTTCTCCTCCATCCGTACCTTGCCTAGCCTCTCC |
| PDE7 | NM_002<br>604.2 | 2210-<br>2310 | GTAGCTCAACAAGGAATAGAGGGAGGAGTGTAATTTTGGTAGCTGGTGTTGA<br>ATAGGGCCTTTGAGAATCAGACTGAACACAGTGAAATATGTGCCCAAA |
| PDK1 | NM_002<br>610.3 | 1170-<br>1270 | TGGATTGCCCATATCACGTCTTTACGCACAATACTTCCAAGGAGACCTGAAGC<br>TGTATTCCCTAGAGGGTTACGGGACAGATGCAGTTATCTACATTAAG |
| PECAM1 | NM_000<br>442.3 | 1365-<br>1465 | ATCTGCACTGCAGGTATTGACAAAGTGGTCAAGAAAAGCAACACAGTCCAGA<br>TAGTCGTATGTGAAATGCTCTCCCAGCCCAGGATTTCTTATGATGCCC |
| PHACTR2 | NM_001<br>100164.1 | 8350-<br>8450 | GGCAGAATGCCACTCTACCCTCAGGTCAATTTTATGGTATATGAAAATGCCAG<br>TAATATTTGTGCCACTTGCCAACTCGGGGGAGGAGGGGCTTTTCCCT |
| PHC1 | NM_004<br>426.2 | 2905-<br>3005 | ATACAGCTCCACCTACACCGGAATTACATGGCATCAACCCTGTGTTCCTGTCC<br>AGTAATCCCAGCCGTTGGAGTGTAGAGGAGGTGTACGAGTTTATTGC |
| POP5 | NM_015<br>918.3 | 560-660 | GCTTCAGGCCCACTTGTTGAACAGAACAATCTGGGTAGCAACAGCATCTTCCA<br>CAGTTTTCCAAACTGGATAGCTGCCAACCAGCAGACATTACCCACTT |
| PPARA | NM_001<br>001928.2 | 5220-<br>5320 | GGGTGTGTTTGCTATACGAACATAATGGACGTGAAGTGGGGCAGAAACCCAG<br>AACTCAGCATTCAAGGATGCCCAGGAGAGCTGTCCCTGTTTTAAAGAG |
| PPP2R1A | NM_014<br>225.3 | 1440-<br>1540 | AACTTAACTCCTTGTGCATGGCCTGGCTTGTGGATCATGTATATGCCATCCGC<br>GAGGCAGCCACCAGCAACCTGAAGAAGCTAGTGGAAAAGTTTGGGAA |
| PRDM1 | NM_182<br>907.1 | 310-410 | CATCCCTGCCAACCAGGAACTTCTTGTGTGGTATTGTCGGGACTTTGCAGAAA<br>GGCTTCACTACCCTTATCCCGGAGAGCTGACAATGATGAATCTCACA |
| PRF1 | NM_005<br>041.3 | 2120-<br>2220 | ACTGTTTTTTCAGGGAGGTGGCTGGGTTTACACGCTAATCCCGATTCACCCTGT<br>CCAAACTGCCTAAGCCCTCCGCCATTCTCAAGCCCTGCAGTCACAGC |
| PROM1 | NM_006<br>017.1 | 925-<br>1025 | AGCCTGCGGTCATCTCTCAATGACCCTCTGTGCTTGGTGCATCCATCAAGTGA<br>AACCTGCAACAGCATCAGATTGTCTCTAAGCCAGCTGAATAGCAACC |
| PTGER2 | NM_000<br>956.2 | 1410-<br>1510 | GTCAGAAGGAGCTACAAAACCTACCCTCAGTGAGCATGGTACTTGGCCTTTG<br>GAGGAACAATCGGCTGCATTGAAGATCCAGCTGCCTATTGATTTAAGC |
| PTK2 | NM_005<br>607.3 | 1005-<br>1105 | GGTTCAAGCTGGATTATTTCAGTGGAACTGGCAATCGGCCCAGAAGAAGGAA<br>TCAGTTACCTAACGGACAAGGGCTGCAATCCCACACATCTTGCTGACT |

207

| | | | |
|---|---|---|---|
| PTPRK | NM_001 135648.1 | 4315-4415 | GTGATCAACCGGATTTTTAGGATATGCAATCTAACAAGACCACAGGAAGGTT ATCTGATGGTGCAACAGTTTCAGTACCTAGGATGGGCTTCTCATCGAG |
| RAC1 | NM_198 829.1 | 1250-1350 | AAAGACCTTCGTCTTTGAGAAGACGGTAGCTTCTGCAGTTAGGAGGTGCAGA CACTTGCTCTCCTATGTAGTTCTCAGATGCGTAAAGCAGAACAGCCTC |
| RAC2 | NM_002 872.3 | 1069-1169 | GCTGCCACAACTTGTGTACCTTCAGGGATGGGGCTCTTACTCCCTCCTGAGGC CAGCTGCTCTAATATCGATGGTCCTGCTTGCCAGAGAGTTCCTCTAC |
| RAP46 | NM_004 323.3 | 1490-1590 | CTCTTGTGATCGTGTAGTCCCATAGCTGTAAAACCAGAATCACCAGGAGGTTG CACCTAGTCAGGAATATTGGGAATGGCCTAGAACAAGGTGTTTGGCA |
| RARA | NM_000 964.2 | 115-215 | AGCCACCTAGCTGGGGCCCATCTAGGAGTGGCATCTTTTTTGGTGCCCTGAAG GCCAGCTCTGGACCTTCCCAGGAAAAGTGCCAGCTCACAGAACTGCT |
| RHOA | NM_001 664.2 | 1230-1330 | GGTACTCTGGTGAGTCACCACTTCAGGGCTTTACTCCGTAACAGATTTTGTTG GCATAGCTCTGGGGTGGGCAGTTTTTTGAAAATGGGCTCAACCAGAA |
| RORA | NM_134 261.2 | 1715-1815 | AAAATTAACCGAGACACTTTATATGGCCCTGCACAGACCTGGAGCGCCACAC ACTGCACATCTTTTGGTGATCGGGGTCAGGCAAAGGAGGGGAAACAAT |
| RORC | NM_001 001523.1 | 1350-1450 | CTCATCAATGCCCATCGGCCAGGGCTCCAAGAGAAAAGGAAAGTAGAACAGC TGCAGTACAATCTGGAGCTGGCCTTTCATCATCATCTCTGCAAGACTC |
| RUNX1 | NM_001 754.4 | 635-735 | CAGCCATGAAGAACCAGGTTGCAAGATTTAATGACCTCAGGTTTGTCGGTCG AAGTGGAAGAGGGAAAAGCTTCACTCTGACCATCACTGTCTTCACAAA |
| RUNX2 | NM_004 348.3 | 1850-1950 | GAAGCCACAGCAGTTCCCCAACTGTTTTGAATTCTAGTGGCAGAATGGATGA ATCTGTTTGGCGACCATATTGAAATTCCTCAGCAGTGGCCCAGTGGTA |
| S100A4 | NM_002 961.2 | 263-363 | CAGGGACAACGAGGTGGACTTCCAAGAGTACTGTGTCTTCCTGTCCTGCATCG CCATGATGTGTAACGAATTCTTTGAAGGCTTCCCAGATAAGCAGCCC |
| SATB1 | NM_001 131010.1 | 1335-1435 | TTCCGAAATCTACCAGTGGGTACGCGATGAACTGAAACGAGCAGGAATCTCC CAGGCGGTATTTGCACGTGTGGCTTTTAACAGAACTCAGGGCTTGCTT |
| SCAP2 | NM_003 930.3 | 3374-3474 | TTTTACAGTTAATCCAGGAGAGGGAGTCCTTTGCCAACTGATGACCAACAGTT CCAAGCCAGATAGTCTCGTGAACAGTGACAATACAGAAATAAGGTGT |
| SCML1 | NM_001 037540.1 | 925-1025 | GCAACGTATGGTTCTTCTTCAGGGCTCTGCCTTGGCAACCCTCGGGCTGACAG CATCCACAACACTTACTCAACTGACCATGCTTCTGCAGCACCACCTT |
| SCML2 | NM_006 089.2 | 360-460 | ATTGGAAGCCCGTGACCCTCGCAATGCCCACTTCAGTATGTATTGCTACGGTTA TTGGAATTACTGGGGCCAGGTTACGGTTACGACTGGATGGTAGTGAC |
| SEL1L | NM_005 065.4 | 980-1080 | GGGCAATCTAATAGCCCACATGGTTTTTGGGTTACAGATACTGGGCTGGCATCG GCGTCCTCCAGAGTTGTGAATCTGCCCTGACTCACTATCGTCTTGTT |
| SELL | NM_000 655.3 | 110-210 | CTCCCTTTGGGCAAGGACCTGAGACCCTTGTGCTAAGTCAAGAGGCTCAATG GGCTGCAGAAGAACTAGAGAAGGACCAAGCAAAGCCATGATATTTCCA |
| SERPINE2 | NM_006 216.2 | 240-340 | CGCTGCCTTCCATCTGCTCCCACTTCAATCCTCTGTCTCTCGAGGAACTAGGCT CCAACACGGGGATCCAGGTTTTCAATCAGATTGTGAAGTCGAGGCC |
| SHP-1 | NM_002 831.5 | 1734-1834 | TGGTGCAGACGGAGGCGCAGTACAAGTTCATCTACGTGGCCATCGCCCAGTT CATTGAAACCACTAAGAAGAAGCTGGAGGTCCTGCAGTCGCAGAAGGG |
| SIT1 | NM_014 450.2 | 720-820 | GCCCCAGCCCCCCGTAGCAGGGGCATGACTGTTTCCCAACCAGCACCCAAAG ACGGGCGCCATTGCCAAGTCACAGGATGTGATCTACCCCGGACTTCCT |
| SLA2 | NM_032 214.2 | 1640-1740 | AAAGGAAAGCTGAGATGATGTCTTACCGTAGCAGCAGATCTTGGATGGTCCA GGCTCTATGTGACCTCCAGAGCAAAGAGAAAGACTTCGGACAGTCTAG |
| SLAMF1 | NM_003 037.2 | 580-680 | GTGTCTCTTGATCCATCCGAAGCAGGCCCTCCACGTTATCTAGGAGATCGCTA CAAGTTTTATCTGGAGAATCTCACCCTGGGGATACGGGAAAGCAGGA |
| SLAMF7 | NM_021 181.3 | 215-315 | GGGCACTATCATAGTGACCCAAAATCGTAATAGGGAGAGAGTAGACTTCCCA GATGGAGGCTACTCCCTGAAGCTCAGCAAACTGAAGAAGAATGACTCA |

| Gene | Accession | Range | Sequence |
|---|---|---|---|
| SLC2A1 | NM_006 516.2 | 2500-2600 | AGGCTCCATTAGGATTTGCCCCTTCCCATCTCTTCCTACCCAACCACTCAAATT AATCTTTCTTTACCTGAGACCAGTTGGGAGCACTGGAGTGCAGGGA |
| SMAD3 | NM_005 902.3 | 4220-4320 | TTAAAGGACAGTTGAAAAGGGCAAGAGGAAACCAGGGCAGTTCTAGAGGAG TGCTGGTGACTGGATAGCAGTTTTAAGTGGCGTTCACCTAGTCAACACG |
| SNAI1 | NM_005 985.2 | 63-163 | GACCACTATGCCGCGCTCTTTCCTCGTCAGGAAGCCCTCCGACCCCAATCGGA AGCCTAACTACAGCGAGCTGCAGGACTCTAATCCAGAGTTTACCTTC |
| SOD1 | NM_000 454.4 | 35-135 | GCCTATAAAGTAGTCGCGGAGACGGGGTGCTGGTTTGCGTCGTAGTCTCCTGC AGCGTCTGGGGTTTCCGTTGCAGTCCTCGGAACCAGGACCTCGGCGT |
| SPI1 | NM_003 120.1 | 730-830 | CTCCGCAGCGGCGACATGAAGGACAGCATCTGGTGGGTGGACAAGGACAAG GGCACCTTCCAGTTCTCGTCCAAGCACAAGGAGGCGCTGGCGCACCGCT |
| STAT1 | NM_007 315.2 | 205-305 | TTTGCTGTATGCCATCCTCGAGAGCTGTCTAGGTTAACGTTCGCACTCTGTGT ATATAACCTCGACAGTCTTGGCACCTAACGTGCTGTGCGTAGCTGCT |
| STAT3 | NM_139 276.2 | 4535-4635 | AGACTTGGGCTTACCATTGGGTTTAAATCATAGGGACCTAGGGCGAGGGTTC AGGGCTTCTCTGGAGCAGATATTGTCAAGTTCATGGCCTTAGGTAGCA |
| STAT4 | NM_003 151.2 | 789-889 | AGACAATGGATCAGAGTGACAAGAATAGTGCCATGGTGAATCAGGAAGTTTT GACACTGCAGGAAATGCTTAACAGCCTCGATTTCAAGAGAAAGGAGGC |
| STAT5A | NM_003 152.2 | 3460-3560 | GAGACAGAGAGAGAGAAAGAGAGAGTGTGTGGGTCTATGTAAATGCATCTGT CCTCATGTGTTGATGTAACCGATTCATCTCTCAGAAGGGAGGCTGGGG |
| STAT5B | NM_012 448.3 | 200-300 | AAGGAGAAGCCCTTCATCAGATGCAAGCGTTATATGGCCAGCATTTTCCCATT GAGGTGCGGCATTATTTATCCCAGTGGATTGAAAGCCAAGCATGGGA |
| Stat6 | NM_003 153.3 | 2030-2130 | AGAACATCCAGCCATTCTCTGCCAAAGACCTGTCCATTCGCTCACTGGGGGAC CGAATCCGGGATCTTGCTCAGCTCAAAAATCTCTATCCCAAGAAGCC |
| STMN1 | NM_203 401.1 | 287-387 | CGTGGGTGGCGGCAGGACTTTCCTTATCCCAGTTGATTGTGCAGAATACACTG CCTGTCGCTTGTCTTCTATTCACCATGGCTTCTTCTGATATCCAGGT |
| TBX21 | NM_013 351.1 | 890-990 | ACACAGGAGCGCACTGGATGCGCCAGGAAGTTTCATTTGGGAAACTAAAGCT CACAAACAACAAGGGGGCGTCCAACAATGTGACCCAGATGATTGTGCT |
| TBXA2R | NM_001 060.3 | 385-485 | CACACGCGCTCCTCCTTCCTCACCTTCCTCTGCGGCCTCGTCCTCACCGACTTC CTGGGGCTGCTGGTGACCGGTACCATCGTGGTGTCCCAGCACGCCG |
| Tcf7 | NM_003 202.2 | 2420-2520 | ATTCCATTTCCAGTTCATCTATGGCAGTCCAGCCAGCTCCTGGGCAGCTTGAG AGGGCAAACCCAAAACCTCATGACAGCCAGAGCCTGTCTTTCAGCAT |
| TDGF1 | NM_003 212.2 | 1567-1667 | AAGGAAAGAAAACATCTTTAAGGGGAGGAACCAGAGTGCTGAAGGAATGGA AGTCCATCTGCGTGTGTGCAGGGAGACTGGGTAGGAAAGAGGAAGCAAA |
| TDO2 | NM_005 651.1 | 0-100 | AAGGTCAATGATAGCATCTGCCTAGAGTCAAACCTCCGTGCTTCTCAGACAGT GCCTTTTCACCATGAGTGGGTGCCCATTTTTAGGAAACAACTTTGGA |
| TEK | NM_000 459.2 | 615-715 | CGAGTTCGAGGAGAGGCAATCAGGATACGAACCATGAAGATGCGTCAACAA GCTTCCTTCCTACCAGCTACTTTAACTATGACTGTGGACAAGGGAGATA |
| TERT | NM_198 253.1 | 2570-2670 | GGCTTCAAGGCTGGGAGGAACATGCGTCGCAAACTCTTTGGGGTCTTGCGGC TGAAGTGTCACAGCCTGTTTCTGGATTTGCAGGTGAACAGCCTCCAGA |
| TF | NM_001 063.2 | 640-740 | CTGCTCCACCCTTAACCAATACTTCGGCTACTCGGGAGCCTTCAAGTGTCTGA AGGATGGTGCTGGGGATGTGGCCTTTGTCAAGCACTCGACTATATTT |
| TFRC | NM_003 234.1 | 1220-1320 | CAGTTTCCACCATCTCGGTCATCAGGATTGCCTAATATACCTGTCCAGACAAT CTCCAGAGCTGCTGCAGAAAAGCTGTTTGGGAATATGGAAGGAGACT |
| TGFA | NM_003 236.2 | 780-880 | TGCCACAGACCTTCCTACTTGGCCTGTAATCACCTGTGCAGCCTTTTGTGGGC CTTCAAAACTCTGTCAAGAACTCCGTCTGCTTGGGGTTATTCAGTGT |
| TGFB1 | NM_000 660.3 | 1260-1360 | TATATGTTCTTCAACACATCAGAGCTCCGAGAAGCGGTACCTGAACCCGTGTT GCTCTCCCGGGCAGAGCTGCGTCTGCTGAGGCTCAAGTTAAAAGTGG |

| Gene | Accession | Position | Sequence |
|---|---|---|---|
| TGFB2 | NM_003238.2 | 1125-1225 | AAGCCAGAGTGCCTGAACAACGGATTGAGCTATATCAGATTCTCAAGTCCAA AGATTTAACATCTCCAACCCAGCGCTACATCGACAGCAAAGTTGTGAA |
| TGFBR1 | NM_004612.2 | 4280-4380 | GGGGAAATACGACTTAGTGAGGCATAGACATCCCTGGTCCATCCTTTCTGTCT CCAGCTGTTTCTTGGAACCTGCTCTCCTGCTTGCTGGTCCCTGACGC |
| TIE1 | NM_005424.2 | 2610-2710 | CATCGGGGAGGGGAACTTCGGCCAGGTCATCCGGGCCATGATCAAGAAGGAC GGGCTGAAGATGAACGCAGCCATCAAAATGCTGAAAGAGTATGCCTCT |
| TLR2 | NM_003264.3 | 180-280 | CTGCTTTCAACTGGTAGTTGTGGGTTGAAGCACTGGACAATGCCACATACTTT GTGGATGGTGTGGGTCTTGGGGGTCATCATCAGCCTCTCCAAGGAAG |
| TLR8 | NM_138636.3 | 2795-2895 | GACAAAAACGTTCTCCTTTGTCTAGAGGAGAGGGATTGGGATCCGGGATTGG CCATCATCGACAACCTCATGCAGAGCATCAACCAAAGCAAGAAAACAG |
| TNF | NM_000594.2 | 1010-1110 | AGCAACAAGACCACCACTTCGAAACCTGGGATTCAGGAATGTGTGGCCTGCA CAGTGAAGTGCTGGCAACCACTAAGAATTCAAACTGGGGCCTCCAGAA |
| TNFRSF18 | NM_004195.2 | 445-545 | AGGGGAAATTCAGTTTTGGCTTCCAGTGTATCGACTGTGCCTCGGGGACCTTC TCCGGGGGCCACGAAGGCCACTGCAAACCTTGGACAGACTGCACCCA |
| TNFRSF1B | NM_001066.2 | 835-935 | CCCAGCTGAAGGGAGCACTGGCGACTTCGCTCTTCCAGTTGGACTGATTGTGG GTGTGACAGCCTTGGGTCTACTAATAATAGGAGTGGTGAACTGTGTC |
| TNFRSF4 | NM_003327.2 | 200-300 | CCGTGCGGGCCGGGCTTCTACAACGACGTGGTCAGCTCCAAGCCGTGCAAGC CCTGCACGTGGTGTAACCTCAGAAGTGGGAGTGAGCGGAAGCAGCTGT |
| TNFRSF7 | NM_001242.4 | 330-430 | CCAGATGTGTGAGCCAGGAACATTCCTCGTGAAGGACTGTGACCAGCATAGA AAGGCTGCTCAGTGTGATCCTTGCATACCGGGGGGTCTCCTTCTCTCCT |
| TNFRSF9 | NM_001561.4 | 255-355 | AGATTTGCAGTCCCTGTCCTCCAAATAGTTTCTCCAGCGCAGGTGGACAAAGG ACCTGTGACATATGCAGGCAGTGTAAAGGTGTTTTCAGGACCAGGAA |
| TNFSF10 | NM_003810.2 | 115-215 | GGGGGGACCCAGCCTGGGACAGACCTGCGTGCTGATCGTGATCTTCACAGTG CTCCTGCAGTCTCTCTGTGTGGCTGTAACTTACGTGTACTTTACCAAC |
| TNFSF14 | NM_003807.2 | 270-370 | ATTTTCAGAAGCCTCTGGAAAGTCGTGCACAGCCCAGGAGTGTTGAGCAATTT CGGTTTCCTCTGAGGTTGAAGGACCCAGGCGTGTCAGCCCTGCTCCA |
| TOX | NM_014729.2 | 3950-4050 | AATGAGCAGCTTTGACTTTGACAGGCGGTTTGTGCAGGAAAGCACAGTGCCG TGTTGTTTACAGCTTTTCTAGAGCAGCTGTGCGACCAGGGTAGAGAGT |
| TP53 | NM_000546.2 | 1330-1430 | GGGGAGCAGGGCTCACTCCAGCCACCTGAAGTCCAAAAAGGGTCAGTCTACC TCCCGCCATAAAAAAACTCATGTTCAAGACAGAAGGGCCTGACTCAGAC |
| TRAF1 | NM_005658.3 | 3735-3835 | CGAGTGATGGGTCTAGGCCCTGAAACTGATGTCCTAGCAATAACCTCTTGATC CCTACTCACCGAGTGTTGAGCCCAAGGGGGGATTTGTAGAACAAGCC |
| TRAF2 | NM_021138.3 | 1325-1425 | GTGGCCCTTCAACCAGAAGGTGACCTTAATGCTGCTCGACCAGAATAACCGG GAGCACGTGATTGACGCCTTCAGGCCCGACGTGACTTCATCCTCTTTT |
| TRAF3 | NM_145725.1 | 1795-1895 | ATATGATGCCCTGCTTCCTTGGCCGTTTAAGCAGAAAGTGACACTCATGCTGA TGGATCAGGGGTCCTCTCGACGTCATTTGGGAGATGCATTCAAGCCC |
| TSLP | NM_033035.3 | 395-495 | CCGTCTCTTGTAGCAATCGGCCACATTGCCTTACTGAAATCCAGAGCCTAACC TTCAATCCCACCGCCGGCTGCGCGTCGCTCGCCAAAGAAATGTTCGC |
| TYK2 | NM_003331.3 | 485-585 | TCATCGCTGACAGCTGAGGAAGTCTGCATCCACATTGCACATAAAGTTGGTAT CACTCCTCCTTGCTTCAATCTCTTTGCCCTCTTCGATGCTCAGGCCC |
| VEGFA | NM_001025366.1 | 1325-1425 | GAGTCCAACATCACCATGCAGATTATGCGGATCAAACCTCACCAAGGCCAGC ACATAGGAGAGATGAGCTTCCTACAGCACAACAAATGTGAATGCAGAC |
| WEE1 | NM_003390.2 | 5-105 | TGCGTTTGAGTTTGCCGCGAGCCGGGCCAATCGGTTTTGCCAACGCATGCCCA CGTGCTGGCGAACAAATGTAAACACGGAGATCGTGTGCCGGGCACTT |
| ZAP70 | NM_001079.3 | 1175-1275 | GGAGCTCAAGGACAAGAAGCTCTTCCTGAAGCGCGATAACCTCCTCATAGCT GACATTGAACTTGGCTGCGGCAACTTTGGCTCAGTGCGCCAGGGCGTG |

| | | | |
|---|---|---|---|
| ZNF516 | NM_014643.2 | 4830-4930 | GGTGGGGGACGGCTTCATATACCTCTTCCTCAGTAATGCAAATGCGAGTTTTTGTGGTGGGGGTTAAGGCCCATAACAAAGGATCTTAAACCATGCAGTG |
| p16 | NM_000077.3 | 975-1075 | AAGCGCACATTCATGTGGGCATTTCTTGCGAGCCTCGCAGCCTCCGGAAGCTGTCGACTTCATGACAAGCATTTTGTGAACTAGGGAAGCTCAGGGGGGT |
| SHP2 | NM_002834.3 | 4650-4750 | TAGTCCCTAGGTTGCTACGGCTTATCATGTGCTTGGTAAAAGGTGATCGCAGGTTCTCAGACGAGTTTACTTTACATGAGATGGAATCAGGCAGAGAGGC |
| CD57/B3GAT1 | NM_018644.3 | 145-245 | CTGGACAGCGACCCCTTCTCAGACTCCAGTTGGGCCGGACTCTCCAAACCTGCTTCCGCAATGGGTGGGTTGTGAGTGCTGGTAATGAGGAGCCGTGGGT |
| CD85/LILRB1 | NM_001081637.1 | 2332-2432 | AGCTGAGAAAACTAAGTCAGAAAGTGCATTAAACTGAATCACAATGTAAATATTACACATCAAGCGATGAAACTGGAAAACTACAAGCCACGAATGAATG |
| Neil1 | NM_024608.2 | 1675-1775 | TTAGCAGGAGGCTCTCCTTGCTTGCACTCACCCTTTCTTATTGTCTTGCCCTGCATCTGGGGGTCTGAATTTTTGGGAGCAGGCAATATCTGAAGGTGCA |
| Neil2 | NM_145043.2 | 2570-2670 | GCCCGGTGGTGTGTAGAGAAAAGCTGCTTGTTTACTCCTTAAGTCAATGTATTGGTGACTGTTGATTTGTTGAACAATTCAGGAATCAAGGGCTGTGGAG |
| PNK | NM_003681.3 | 580-680 | TCCCGGAGGACCTCCTTCCCGTCTACAAAGAAAAAGTGGTGCCGCTTGCAGACATTATCACGCCCAACCAGTTTGAGGCCGAGTTACTGAGTGGCCGGAA |
| POLR2A | NM_000937.2 | 3775-3875 | TTCCAAGAAGCCAAAGACTCCTTCGCTTACTGTCTTCCTGTTGGGCCAGTCCGCTCGAGATGCTGAGAGAGCCAAGGATATTCTGTGCCGTCTGGAGCAT |
| POLR1B | NM_019014.3 | 3320-3420 | GGAGAACTCGGCCTTAGAATACTTTGGTGAGATGTTAAAGGCTGCTGGCTACAATTTCTATGGCACCGAGAGGTTATATAGTGGCATCAGTGGGCTAGAA |
| IL-1alpha | NM_000575.3 | 1085-1185 | ACTCCATGAAGGCTGCATGGATCAATCTGTGTCTCTGAGTATCTCTGAAACCTCTAAAACATCCAAGCTTACCTTCAAGGAGAGCATGGTGGTAGTAGCA |
| IL-1beta | NM_000576.2 | 840-940 | GGGACCAAAGGCGGCCAGGATATAACTGACTTCACCATGCAATTTGTGTCTTCCTAAAGAGAGCTGTACCCAGAGAGTCCTGTGCTGAATGTGGACTCAA |
| IL-12p40 | NM_002187.2 | 1435-1535 | GCAAGGCTGCAAGTACATCAGTTTTATGACAATCAGGAAGAATGCAGTGTTCTGATACCAGTGCCATCATACACTTGTGATGGATGGGAACGCAAGAGAT |
| Raf-1 | NM_002880.2 | 1990-2090 | CCTATGGCATCGTATTGTATGAACTGATGACGGGGGAGCTTCCTTATTCTCACATCAACAACCGAGATCAGATCATCTTCATGGTGGGCCGAGGATATGC |
| IL-23p19 | NM_016584.2 | 411-511 | CAGGGACAACAGTCAGTTCTGCTTGCAAAGGATCCACCAGGGTCTGATTTTTTATGAGAAGCTGCTAGGATCGGATATTTTCACAGGGGAGCCTTCTCTG |
| gBAD-1R_scfv | SCFV001.1 | 1-101 | AGACAGACACCCTGCTCCTCTGGGTGTCCGGCACCTGTGGCGACATCGTGATGAGCAGAAGCCCCAGCAGCCTGGCCGTGTCCGTGGGCGAGAAAGTGAC |
| CD20_scfv_rutuximab | SCFV002.1 | 8-108 | GCTGTCCCAGAGCCCCGCCATCCTGAGCGCCAGCCCTGGCGAGAAGGTGACCATGACCTGCCGGGCCAGCAGCTCTGTGAGCTACATGCACTGGTATCAG |
| c-MET_scfv | SCFV004.1 | 138-238 | CTGATCTACGCCGCCAGCAGCCTGAAGAGCGGCGTGCCCAGCCGGTTTAGCGGCTCTGGCTCTGGCGCCGACTTCACCCTGACCATCAGCAGCCTGCAGC |
| CD45R_scfv | SCFV006.1 | 222-322 | TTCACCCTGAACATCCACCCCGTGGAGGAAGAGGACGCCGCCACCTACTACTGCCAGCACAGCAGAGAGCTGCCCTTCACCTTCGGCTCCGGCACCAAGC |
| Thymidine_kinase | SCFV007.1 | 100-200 | TCTACGTACCCGAGCCGATGACTTACTGGCAGGTGCTGGGGGGCTTCCGAGACAATCGCGAACATCTACACCACACAACACCGCCTCGACCAGGGTGAGAT |
| CD56R_scfv | SCFV008.1 | 197-297 | ATTCAGCGGCTCTGGCTCCGGCACCGACTTCACTCTGATGATCTCTCGGGTGGAGGCCGAGGACCTGGGCGTGTACTACTGCTTTCAGGGCAGCCACGTG |
| Human_CD19R_scfv | SCFV009.1 | 215-315 | CTTCACCATCAGCAGCCTGCAGCCCGAGGACATCGCCACCTACTACTGCCAGCAGTACCAGAGCCTGCCCTACACCTTCGGCCAGGGCACCAAGCTGCAG |
| DECTIN-1R | SCFV010.1 | 270-370 | CTGAAGATCGACAGCAGCAACGAGCTGGGCTTCATCGTGAAGCAGGTGTCCAGCCAGCCCGACAACTCCTTCTGGATCGGCCTGAGCAGGCCCCAGACCG |

| | | | |
|---|---|---|---|
| HERV-K_6H5_scfv | SCFV012.1 | 137-237 | CGGCGGCACCAGCTACAACCAGAAGTTCAAGGACAAGGCCATCCTGACCGTG GACAAGAGCAGCAGCACCGCCTACATGGAACTGCGGAGCCTGACCAGC |
| CD19R_scfv | SCFV013.1 | 204-304 | GGCACCGACTACAGCCTGACCATCTCCAACCTGGAGCAGGAGGACATCGCCA CCTACTTTTGCCAGCAGGGCAACACACTGCCCTACACCTTTGGCGGCG |
| HER2_scfv | SCFV014.1 | 64-164 | CCTGCAGCGCCAGCAGCAGCGTGTCCTACATGCACTGGTATCAGCAGAAGTC CGGCACTAGCCCCAAGCGGTGGATCTACGACACCTACAAGCTCGCCAG |
| EGFR_scfv_NIMO_CAR | SCFV015.1 | 7-107 | AGATGACCCAGAGCCCTAGCAGCCTGAGCGCCAGCGTGGGCGACAGAGTGA CCATCACCTGCCGGTCCAGCCAGAACATCGTGCACAGCAACGGCAACAC |
| RPL27 | NM_000988.3 | 23-123 | GGGCCGGGTGGTTGCTGCCGAAATGGGCAAGTTCATGAAACCTGGGAAGGTG GTGCTTGTCCTGGCTGGACGCTACTCCGGACGCAAAGCTGTCATCGTG |
| OAZ1 | NM_004152.2 | 313-413 | GGTGGGCGAGGGAATAGTCAGAGGGATCACAATCTTTCAGCTAACTTATTCT ACTCCGATGATCGGCTGAATGTAACAGAGGAACTAACGTCCAACGACA |
| GABPa | NM_002040.3 | 1160-1260 | GACCAAGTCCTGCATTGGGTGGTTTGGGTAATGAAGGAATTCAGCATGACCG ATATAGACCTCACCACACTCAACATTTCGGGGAGAGAATTATGTAGTC |
| XBP-1 | NM_005080.2 | 440-540 | GGAGTTAAGACAGCGCTTGGGGATGGATGCCCTGGTTGCTGAAGAGGAGGCG GAAGCCAAGGGGAATGAAGTGAGGCCAGTGGCCGGGTCTGCTGAGTCC |
| MBD2 | NM_003927.3 | 2015-2115 | ATTTACATTCAACTCTGATCCCTGGGCCTTAGGTTTGACATGGAGGTGGAGGA AGATAGCGCATATATTTGCAGTATGAACTATTGCCTCTGGACGTTGT |
| Bcl6b | NM_181844.3 | 2135-2235 | CTTTATTTGTTCTAGGGCAGCTCTGGGAACATGCGGGATTGTGGAATTGGGTC AGGAACCCTCTCTGGTATTCTGGATGTTGTAGGTTCTCTAGCAGTCT |
| TSLP-R | NM_022148.2 | 1420-1520 | CAAGGCAGCACGTCCAAAATGCTGTAAAACCATCTTCCCACTCTGTGAGTCCC CAGTTCCGTCCATGTACCTGTTCCATAGCATTGGATTCTCGGAGGAT |
| BTLA | NM_001085357.1 | 890-990 | GCACCAACAGAATATGCATCCATATGTGTGAGGAGTTAAGTCTGTTTCTGACT CCAACAGGGACCATTGAATGATCAGCATGTTGACATCATTGTCTGGG |
| HVEM | NM_003820.2 | 916-1016 | CTCAGGGAGCCTCGTCATCGTCATTGTTTGCTCCACAGTTGGCCTAATCATAT GTGTGAAAAGAAGAAAGCCAAGGGGTGATGTAGTCAAGGTGATCGTC |
| LTbR | NM_002342.1 | 1435-1535 | CTAACAGGGGCCCAAGGAACCAATTTATCACCCATGACTGACGGAGTCTGAG AAAAGGCAGAAGAAGGGGGGGCACAAGGGCACTTTCTCCCTTGAGGCTG |
| CD43 | NM_001030288.1 | 2798-2898 | AAGCCAGGCTTCATGGAAAGATCGTATGTGTGACCCAAATATGAGTTCTTCA GCTCAGCCATGGTAATCCCTTCCTTGAAGTCTCCATTTCTGCAGTACA |
| mTOR | NM_004958.2 | 5095-5195 | TTAGTGTTGCTCCTGGGAGTTGATCCGTCTCGGCAACTTGACCATCCTCTGCC AACAGTTCACCCTCAGGTGACCTATGCCTACATGAAAAACATGTGGA |
| AMPK | NM_006252.2 | 975-1075 | ATAGTGGTGACCCTCAAGACCAGCTTGCAGTGGCTTATCATCTTATCATTGAC AATCGGAGAATAATGAACCAAGCCAGTGAGTTCTACCTCGCCTCTAG |
| SIP1 | NM_001009182.1 | 537-637 | ACAAGCAACAGTAACTAGTGTCTTGGAATATCTGAGTAATTGGTTTGGAGAA AGAGACTTTACTCCAGAATTGGGAAGATGGCTTTATGCTTTATTGGCT |
| EphA2 | NM_004431.2 | 1525-1625 | GAGCCGAGTGTGGAAGTACGAGGTCACTTACCGCAAGAAGGGAGACTCCAAC AGCTACAATGTGCGCCGCACCGAGGGTTTCTCCGTGACCCTGGACGAC |
| CD254 | NM_003701.2 | 490-590 | TACCTGATTCATGTAGGAGAATTAAACAGGCCTTTCAAGGAGCTGTGCAAAA GGAATTACAACATATCGTTGGATCACAGCACATCAGAGCAGAGAAAGC |
| BCLxL | NM_001191.2 | 260-360 | ATCTTGGCTTTGGATCTTAGAAGAGAATCACTAACCAGAGACGAGACTCAGT GAGTGAGCAGGTGTTTTGGACAATGGACTGGTTGAGCCCATCCCTATT |
| Xbp1 | NM_001079539.1 | 935-1035 | ATTCATTGTCTCAGTGAAGGAAGAACCTGTAGAAGATGACCTCGTTCCGGAG CTGGGTATCTCAAATCTGCTTTCATCCAGCCACTGCCCAAAGCCATCT |
| IL27 | NM_145659.3 | 143-243 | CAGGAGCTGCGGAGGGAGTTCACAGTCAGCCTGCATCTCGCCAGGAAGCTGC TCTCCGAGGTTCGGGGCCAGGCCCACCGCTTTGCGGAATCTCACCTGC |

| IKZF2 | NM_001 079526.1 | 945-1045 | CCATGTACCTCCTATGGAAGATTGTAAGGAACAAGAGCCTATTATGGACAAC AATATTTCTCTGGTGCCTTTTGAGAGACCTGCTGTCATAGAGAAGCTC |
|---|---|---|---|
| GNLY | NM_006 433.2 | 305-405 | CAGGAGCTGGGCCGTGACTACAGGACCTGTCTGACGATAGTCCAAAAACTGA AGAAGATGGTGGATAAGCCCACCCAGAGAAGTGTTTCCAATGCTGCGA |
| NFkB | NM_001 165412.1 | 2305-2405 | CTTGGGTAACTCTGTTTTGCACCTAGCTGCCAAAGAAGGACATGATAAAGTTC TCAGTATCTTACTCAAGCACAAAAAGGCAGCACTACTTCTTGACCAC |
| GADD45alp ha | NM_001 924.2 | 865-965 | GTTACTCCCTACACTGATGCAAGGATTACAGAAACTGATGCCAAGGGGCTGA GTGAGTTCAACTACATGTTCTGGGGGCCCGGAGATAGATGACTTTGCA |
| GADD45bet a | NM_015 675.2 | 365-465 | TGTGGACCCAGACAGCGTGGTCCTCTGCCTCTTGGCCATTGACGAGGAGGAG GAGGATGACATCGCCCTGCAAATCCACTTCACGCTCATCCAGTCCTTC |
| ATF3 | NM_001 030287.2 | 600-700 | GGCTCAGAATGGGAGGACTCCAGAAGATGAGAGAAACCTCTTTATCCAACAG ATAAAAGAAGGAACATTGCAGAGCTAAGCAGTCGTGGTATGGGGGCGA |
| MAD | NM_002 357.2 | 880-980 | GAGAATAAAGCTGCAGGACAGTCACAAGGCGTGTCTTGGTCTCTAAGAGAGT GGGCACTGCGGCTGTCTCCTTGAAGGTTCTCCCTGTTGGTTCTGATTA |
| Crem | NM_001 881.2 | 260-360 | CTCCACCTCCTCGCGTCCGTAATCAGTGACGAGGTCCGCTACGTAAATCCCTT TGCGGCGGACAAATGACCATGGAAACAGTTGAATCCCAGCATGATGG |
| SOCS1 | NM_003 745.1 | 1025-1125 | TTAACTGTATCTGGAGCCAGGACCTGAACTCGCACCTCCTACCTCTTCATGTT TACATATACCCAGTATCTTTGCACAAACCAGGGGTTGGGGGGAGGGTC |
| SOCS3 | NM_003 955.3 | 1870-1970 | GGAGGATGGAGGAGACGGGACATCTTTCACCTCAGGCTCCTGGTAGAGAAGA CAGGGGATTCTACTCTGTGCCTCCTGACTATGTCTGGCTAAGAGATTC |
| DUSP16 | NM_030 640.2 | 615-715 | ATGGGTTTAACTCTCCTTTTGCCAGTCACCACCAGCCTGACCTCATACACTTTT AGTACAATGGAGTGGCTGAGCCTTTGAGCACACCACCATTACATCA |
| Rps13 | NM_001 017.2 | 331-431 | GCATCTTGAGAGGAACAGAAAGGATAAGGATGCTAAATTCCGTCTGATTCTA ATAGAGAGCCGGATTCACCGTTTGGCTCGATATTATAAGACCAAGCGA |
| TBP | NM_003 194.3 | 25-125 | CGCCGGCTGTTTAACTTCGCTTCCGCTGGCCCATAGTGATCTTTGCAGTGACC CAGCAGCATCACTGTTTCTTGGCGTGTGAAGATAACCCAAGGAATTG |
| G6PD | NM_000 402.2 | 1155-1255 | ACAACATCGCCTGCGTTATCCTCACCTTCAAGGAGCCCTTTGGCACTGAGGGT CGCGGGGGCTATTTCGATGAATTTGGGATCATCCGGGACGTGATGCA |
| Rbpms | NM_001 008710.1 | 842-942 | AAACAGCCTGTAGGTTTTGTCAGTTTTGACAGTCGCTCAGAAGCAGAGGCTGC AAAGAATGCTTTGAATGGCATCCGCTTCGATCCTGAAATTCCGCAAA |
| KLF7 | NM_001 270943.1 | 1546-1646 | GTACTATTGAGATCTTTCGCGTCGATCCCAACGGCCTTAGCGGCGGCAGACTG GAATAACACCTTACACCTTTCTGGCCTGCATTTCTGTAGACTTCACT |
| Vax2 | NM_012 476.2 | 871-971 | CAGCGCCAGCAGCTGCAAGAAAGCTAACACTTAAGACTCCCACCCTGTGACA CTGAGTCCCGAGCACAGCACCTTCCCAGTCTCCTGTGCCCCAGCGGAC |
| RUNX3 | NM_004 350.1 | 2085-2185 | GTGGTCTCATAATTCCATTTGTGGAGAGAACAGGAGGGCCAGATAGATAGGT CCTAGCAGAAGGCATTGAGGTGAGGGATCATTTTGGGTCAGACATCAA |
| ERK | NM_017 449.2 | 785-885 | CAAAGCAGGCTTCGAGGCCGTTGAGAATGGCACCGTCTGCCGAGGTTGTCCA TCTGGGACTTTCAAGGCCAACCAAGGGGATGAGGCCTGTACCCACTGT |
| ITCH | NM_031 483.4 | 155-255 | ACTGTGAGAACTTCAGGTTTTCCAACCTATTGGTGGTATGTCTGACAGTGGAT CACAACTTGGTTCAATGGGTAGCCTCACCATGAAATCACAGCTTCAG |
| CBLB | NM_170 662.3 | 3195-3295 | TAATGTCGAAGTTGCCCGGAGCATCCTCCGAGAATTTGCCTTCCCTCCTCCAG TATCCCCACGTCTAAATCTATAGCAGCCAGAACTGTAGACACCAAAA |
| DGKA | NM_001 345.4 | 1375-1475 | TTCCTAACACCCACCCACTTCTCGTCTTTGTCAATCCTAAGAGTGGCGGGAAG CAGGGGCAAAGGGTGCTCTGGAAGTTCCAGTATATATTAAACCCTCG |
| LTA | NM_000 595.2 | 885-985 | CTGATCAAGTCACCGGAGCTTTCAAAGAAGGAATTCTAGGCATCCCAGGGGA CCACACCTCCCTGAACCATCCCTGATGTCTGTCTGGCTGAGGATTTCA |

| | | | |
|---|---|---|---|
| FoxP1 | NM_032 682.5 | 6758-6858 | CCTGAAAATCAGATTTACAATGCTGAAGGCATTTCTTGGGCCCAGTGTAGCTC ACGCAATCTCTGCTACCCATAAGCCTTGATGAAGATGATACAGTCCG |
| CD223 (LAG3) | NM_002 286.5 | 1735-1835 | CTTTTGGTGACTGGAGCCTTTGGCTTTCACCTTTGGAGAAGACAGTGGCGACC AAGACGATTTTCTGCCTTAGAGCAAGGGATTCACCCTCCGCAGGCTC |
| CD118 | NM_002 310.3 | 2995-3095 | CCTATTGTCCACCCATCATTGAGGAAGAAATACCAAACCCAGCCGCAGATGA AGCTGGAGGGACTGCACAGGTTATTTACATTGATGTTCAGTCGATGTA |
| Txk | NM_003 328.1 | 800-900 | ATGACTCGTCTCCGATATCCAGTTGGGCTGATGGGCAGTTGTTTACCAGCCAC AGCTGGGTTTAGCTACGAAAAGTGGGAGATAGATCCATCTGAGTTGG |
| Prkcq | NM_006 257.2 | 1325-1425 | GATGGACGATGATGTTGAGTGCACGATGGTAGAGAAGAGAGTTCTTTCCTTG GCCTGGGAGCATCCGTTTCTGACGCACATGTTTTGTACATTCCAGACC |
| STS2 (Ubash3a) | NM_001 001895.1 | 1970-2070 | GAGATGCTGCTGTTTCCAGAGGCGTCTTAGTCTCACCCAATGTGATTTGTAGA AGCACGAGACGCACTTTTATATCCCGGAATATTTCCCTCCGGCTTTC |
| RNF125 | NM_017 831.3 | 790-890 | GCAAGGTGTGTATGTCCCTTTTGTCAGAGGGAACTGTATGAAGACAGCTTGCT GGATCATTGTATTACTCATCACAGATCGGAACGGAGGCCTGTGTTCT |
| Lat | NM_001 014987.1 | 1290-1390 | TGTGTAATAGAATAAAGGCCTGCGTGTGTCTGTGTTGAGCGTGCGTCTGTGTG TGCCTGTGTGCGAGTCTGAGTCAGAGATTTGGAGATGTCTCTGTGTG |
| Skap1 | NM_003 726.3 | 1360-1460 | AAGTGGGAAGAGGCACGTTCATCAAACCTGTTACTAAACCAGCCTAGTCATA GCTCATCCCCATCTCTAAATGTGTCCACACAACCACATCTGCCTTTTC |
| Dok2 | NM_003 974.2 | 650-750 | GCCAGGGACCCAGCTGTACGACTGGCCCTACAGGTTTCTGCGGCGCTTTGGGC GGGACAAGGTAACCTTTTCCTTTGAGGCAGGCCGTCGCTGCGTCTCT |
| Axin2 | NM_004 655.3 | 1035-1135 | CTTGTCCAGCAAAACTCTGAGGGCCACGGCGAGTGTGAGGTCCACGGAAACT GTTGACAGTGGATACAGGTCCTTCAAGAGGAGCGATCCTGTTAATCCT |
| Sh2d2a | NM_001 161443.1 | 341-441 | TGCTGGAGCCCAAGCCTCAGGGGTGCTACTTGGTGCGGTTCAGCGAGAGCGC GGTGACCTTCGTGCTGACTTACAGGAGCCGGACTTGCTGCCGCCACTT |
| Klra5 (Ly49E) | NR_028 045.1 | 414-514 | CCTTCAGAGTCACAGAATAGATTAAGGCCTGATGATACTCAAAGGCCTGGGA AAACTGATGACAAAGAATTTTCAGTGCCCTGGCACCTCATTGCAGTGA |
| CD7 | NM_006 137.6 | 440-540 | CCTACACCTGCCAGGCCATCACGGAGGTCAATGTCTACGGCTCCGGCACCCT GGTCCTGGTGACAGAGGAACAGTCCCAAGGATGGCACAGATGCTCGGA |
| CD11c | NM_000 887.3 | 700-800 | CCCCTCAGCCTGTTGGCTTCTGTTCACCAGCTGCAAGGGTTTACATACACGGC CACCGCCATCCAAAATGTCGTGCACCGATTGTTCCATGCCTCATATG |
| Syk | NM_003 177.3 | 1685-1785 | CGGACTCTCCAAAGCACTGCGTGCTGATGAAAACTACTACAAGGCCCAGACC CATGGAAAGTGGCCTGTCAAGTGGTACGCTCCGGAATGCATCAACTAC |
| Lyn | NM_002 350.1 | 1285-1385 | TCCTGAAGAGCGATGAAGGTGGCAAAGTGCTGCTTCCAAAGCTCATTGACTTT TCTGCTCAGATTGCAGAGGGAATGGCATACATCGAGCGGAAGAACTA |
| Lat2 | NM_014 146.3 | 1863-1963 | TGCAGAGCTGATTAAACAGTGTTGTGACTGTCTCATGGGAAGAGCTGGGGCC CAGAGGGACCTTGAGTCAGAAATGTTGCCAGAAAAAGTATCTCCTCCA |
| Clnk | NM_052 964.2 | 1108-1208 | GAAGGAGAACAAGGATGGTAGTTTCTTGGTCCGAGATTGTTCCACAAAATCC AAGGAAGAGCCCTATGTTTTGGCTGTGTTTTATGAGAACAAAGTCTAC |
| Car2 | NM_000 067.2 | 575-675 | AGCTGTGCAGCAACCTGATGGACTGGCCGTTCTAGGTATTTTTTTGAAGGTTG GCAGCGCTAAACCGGGCCTTCAGAAAGTTGTTGATGTGCTGGATTCC |
| Fgl2 | NM_006 682.2 | 250-350 | CAATTCAGCAGGATCGAGGAGGTGTTCAAAGAAGTCCAAAACCTCAAGGAAA TCGTAAATAGTCTAAAGAAATCTTGCCAAGACTGCAAGCTGCAGGCTG |
| cathepsinC | NM_001 114173.1 | 260-360 | TGCTCGGTTATGGGACCACAAGAAAAAAAAGTAGTGGTGTACCTTCAGAAGC TGGATACAGCATATGATGACCTTGGCAATTCTGGCCATTTCACCATCA |
| CathepsinD | NM_001 909.3 | 1495-1595 | GAAGCCGGCGGCCCAAGCCCGACTTGCTGTTTTGTTCTGTGGTTTTCCCCTCC CTGGGGTTCAGAAATGCTGCCTGCCTGTCTGTCTCTCCATCTGTTTGG |

| Gene | Accession | Position | Sequence |
|---|---|---|---|
| Rab31 | NM_006 868.3 | 3800-3900 | TTTTGTAAAGAGCTTCCATCTGGGCTGGACCCAGTTCTTGCACATACAAGACA CCGCTGCAGTCAGCTAGGACCTTTCCGCCATGTATTCTATTCTGTAG |
| Spry2 | NM_005 842.2 | 85-185 | AAAGAGGAAATACTCCGCGTGCGCTTGTAGAAGGGGAGTCGTCTCCAGCTCC GAACCCCGGAGTGTTCATCAGCGGGGAATCTGGCTCCGAATTCTCTTT |
| S100A6 | NM_014 624.3 | 539-639 | TTCCTGGGGGCCTTGGCTTTGATCTACAATGAAGCCCTCAAGGGCTGAAAATA AATAGGGAAGATGGAGACACCCTCTGGGGGTCCTCTCTGAGTCAAAT |
| Lgals1 | NM_002 305.3 | 60-160 | GGTGCGCCTGCCCGGGAACATCCTCCTGGACTCAATCATGGCTTGTGGTCTGG TCGCCAGCAACCTGAATCTCAAACCTGGAGAGTGCCTTCGAGTGCGA |
| Hmgb2 | NM_001 130688.1 | 125-225 | CTGTCAACATGGGTAAAGGAGACCCCAACAAGCCGCGGGGCAAAATGTCCTC GTACGCCTTCTTCGTGCAGACCTGCCGGGAAGAGCACAAGAAGAAACA |
| HopX | NM_001 145460.1 | 1117-1217 | AACAATAGGAAGCTATGTGTATCTTCTGTGTAAAGCAGTGGCTTCACTGGAA AAATGGTGTGGCTAGCATTTCCCTTTGAGTCATGATGACAGATGGTGT |
| Dock5 | NM_024 940.6 | 630-730 | TGCGAGATGACAATGGGAACATCCTAGACCCTGACGAAACCAGCACCATTGC CCTCTTCAAGGCCCATGAGGTGGCCTCCAAAAGGATTGAGGAAAGAT |
| Ptpn4 | NM_002 830.2 | 705-805 | TCGAGGCTTTTTTTTCTCCAGCCGAGAGGACGCGGCTGTGATATACGAAGACTT TGTGTGGACAGTAATGACCTCACGTTTCCGATTGCCTGCTGGCAGAA |
| PLZF | NM_006 006.4 | 1585-1685 | TCCTGGATAGTTTGCGGCTGAGAATGCACTTACTGGCTCATTCAGCGGGTGCC AAAGCCTTTGTCTGTGATCAGTGCGGTGCACAGTTTTCGAAGGAGGA |
| Foxo1 | NM_002 015.3 | 1526-1626 | TCTCATCACCAACATCATTAACTGTTTCGACCCAGTCCTCACCTGGCACCATG ATGCAGCAGACGCCGTGCTACTCGTTGCGCCACCAAACACCAGTTT |
| Foxo3 | NM_001 455.2 | 1860-1960 | CCGGAACGTGATGCTTCGCAATGATCCGATGATGTCCTTTGCTGCCCAGCCTA ACCAGGGAAGTTTGGTCAATCAGAACTTGCTCCACCACCAGCACCAA |
| ID3 | NM_002 167.3 | 195-295 | AGGAAGCCTGTTTGCAATTTAAGCGGGCTGTGAACGCCCAGGGCCGGCGGGG GCAGGGCCGAGGCGGGCCATTTTGAATAAAGAGGCGTGCCTTCCAGGC |
| ZEB2 | NM_014 795.2 | 20-120 | TCCCAGAGAGAAACTTGGCGATCACGTTTTCACATGATGCTCACGCTCAGGGC GCTTCAATTATCCCTCCCCACAAAGATAGGTGGCGCGTGTTTCAGGG |
| SMAD4 | NM_005 359.3 | 1370-1470 | AGGTTGCACATAGGCAAAGGTGTGCAGTTGGAATGTAAAGGTGAAGGTGATG TTTGGGTCAGGTGCCTTAGTGACCACGCGGTCTTTGTACAGAGTTACT |
| YAP | NM_139 118.2 | 755-855 | ATGGGAGCTATGCAGCTGATTGAAGACTTCAGCACACATGTCAGCATTGACT GCAGCCCTCATAAAACTGTCAAGAAGACTGCCAATGAATTTCCCTGTT |
| E2A | NM_003 200.2 | 4325-4425 | ATACGTGTCAACACAGCTGGCTGGATGATTGGGACTTTAAAACGACCCTCTTT CAGGTGGATTCAGAGACCTGTCCTGTATATAACAGCACTGTAGCAAT |
| Nanog | NM_024 865.2 | 1100-1200 | CTACTCCATGAACATGCAACCTGAAGACGTGTGAAGATGAGTGAAACTGATA TTACTCAATTTCAGTCTGGACACTGGCTGAATCCTTCCTCTCCCCTCC |
| OCT4 | NM_002 701.4 | 1225-1325 | AAGTTCTTCATTCACTAAGGAAGGAATTGGGAACACAAAGGGTGGGGGCAGG GGAGTTTGGGGCAACTGGTTGGAGGGAAGGTGAAGTTCAATGATGCTC |
| Sox2 | NM_003 106.2 | 151-251 | CTTAAGCCTTTCCAAAAAATAATAATAACAATCATCGGCGGCGGCAGGATCG GCCAGAGGAGGAGGGAAGCGCTTTTTTTGATCCTGATTCCAGTTTGCC |
| TAL1 | NM_003 189.2 | 4635-4735 | ACAGCATCTGTAGTCAGCCGACAACTATTTCGGCCTTTTGGGGGTGGGTCTGG CCGTACTTGTGATTTCGATGGTACGTGACCCTCTGCTGAAGACTTGC |
| ELF1 | NM_032 377.3 | 125-225 | AGACCCAGTTCACCTGCCCCTTCTGCAACCACGAGAAATCCTGTGATGTGAAA ATGGACCGTGCCCGCAACACCGGAGTCATCTCTTGTACCGTGTGCCT |
| SOX13 | NM_005 686.2 | 3039-3139 | ATTTATTGAGTGCCCACTACGTGCCAGGCACTGTTGCTGAGTTCCTGTGGGTG TGTCTCTCGATGCCACTCCTGCTTCTCTGGGGGCCTCTTTCTGTGCT |
| Nrp1 | NM_003 873.5 | 370-470 | GCCTCGCTGCTTTCTTTTCTCCAAGACGGGCTGAGGATTGTACAGCTCTAGGC GGAGTTGGGGCTCTTCGGATCGCTTAGATTCTCCTCTTTGCTGCATT |

| | | | |
|---|---|---|---|
| Blk | NM_001 715.2 | 990-1090 | AGCTTCTTGCTCCAATCAACAAGGCCGGCTCCTTTCTTATCAGAGAGAGTGAA ACCAACAAAGGTGCCTTCTCCCTGTCTGTGAAGGATGTCACCACCCA |
| CCR10 | NM_001 296.3 | 1345-1445 | GAACAGATGGGAACCAGCTCAATTGGGTGTCCACTCAAAGTGCTCTCTCCAG GGGCCTCAGTGACTGTGTTGCTAAACCCAGTGGTCAGTTCTCAGTTCT |
| ITGB7 | NM_000 889.1 | 1278-1378 | CAACGTGGTACAGCTCATCATGGATGCTTATAATAGCCTGTCTTCCACCGTGA CCCTTGAACACTCTTCACTCCCTCCTGGGGTCCACATTTCTTACGAA |
| Sox5 | NM_152 989.2 | 1885-1985 | TAGCCATGCAATGATGGATTTCAATCTGAGTGGAGATTCTGATGGAAGTGCTG GAGTCTCAGAGTCAAGAATTTATAGGGAATCCCGAGGGCGTGGTAGC |
| Bcl11b | NM_022 898.1 | 3420-3520 | GAGATGTAGCACTCATGTCGTCCCGAGTCAAGCGGCCTTTTCTGTGTTGATTT CGGCTTTCATATTACATAAGGGAAACCTTGAGTGGTGGTGCTGGGGG |
| SOX4 | NM_003 107.2 | 3040-3140 | GTTCACGGTCAAACTGAAATGGATTTGCACGTTGGGGAGCTGGCGGCGGCGG CTGCTGGGCCTCCGCCTTCTTTTCTACGTGAAATCAGTGAGGTGAGAC |
| Tcf12 | NM_207 037.1 | 1105-1205 | CACATGACCGCTTGAGTTATCCTCCACACTCAGTTTCACCAACAGACATAAAC ACGAGTCTTCCACCAATGTCCAGCTTTCATCGCGGCAGTACCAGCAG |
| Dapl 1 | NM_001 017920.2 | 190-290 | CGAGAAAACAAGTGCCATTGCAAATGTTGCCAAAATACAGACACTGGATGCC CTGAATGACGCACTGGAGAAGCTCAACTATAAATTTCCAGCAACAGTG |
| Trf | NM_003 218.3 | 1037-1137 | CTGAAAGCAGAATACCTGTTTCAAAGAGTCAGCCGGTAACTCCTGAAAAACA TCGAGCTAGAAAAAGACAGGCATGGCTTTGGGAAGAAGACAAGAATTT |
| Cpt1 | NM_020 244.2 | 1303-1403 | GATATGGTGATATACTTTAGTGCTTTGTGCCTGCAAATTTCAAGACACCTTCA TCTAAATATATTCAAGACTGCATGTCATCAAGCACCTGAACAGGTTC |
| Bim | NM_138 621.4 | 257-357 | CGGACTGAGAAACGCAAGAAAAAAAGACCAAATGGCAAAGCAACCTTCTGA TGTAAGTTCTGAGTGTGACCGAGAAGGTAGACAATTGCAGCCTGCGGAG |
| C-flip | NM_001 127183.1 | 653-753 | TAGAGTGCTGATGGCAGAGATTGGTGAGGATTTGGATAAATCTGATGTGTCCT CATTAATTTTCCTCATGAAGGATTACATGGGCCGAGGCAAGATAAGC |

**Appendix B. Antibodies Used in Dissertation**

| Antibody specificity | Clone | Vendor |
|---|---|---|
| Fc* | H10104 | Invitrogen |
| anti-CD19scFv mAb** | 136.20.1 | Cooper Lab |
| ROR1 | 4A5 | Kipps, TJ Lab (UCSD) |
| CD3 | SK7 | BD Biosciences |
| CD4 | RPA-T4 | BD Biosciences |
| CD8 | RPA-T8 | BD Biosciences |
| CD19 | HIB19 | BD Biosciences |
| CD25 | M-A251 | BD Biosciences |
| CD27 | M-T271 | BD Biosciences |
| CD28 | L293 | BD Biosciences |
| CD32 | FLI8.26 (2003) | BD Biosciences |
| CD38 | HB7 | BD Biosciences |
| CD45RA | HI100 | BD Biosciences |
| CD45RO | UCHL1 | BD Biosciences |
| CD56 | B159 | BD Biosciences |
| CD57 | NK-1 | BD Biosciences |
| CD62L | Dreg 56 | BD Biosciences |

| | | |
|---|---|---|
| CD64 | 10.1 | BD Biosciences |
| CD86 | 2331 FUN-1 | BD Biosciences |
| CD95 | DX2 | BD Biosciences |
| CD122 | TM-Beta 1 | BD Biosciences |
| CD127 | HIL-7R-M21 | BD Biosciences |
| CD137 | 4B4-1 | BD Biosciences |
| CD137L | C65-485 | BD Biosciences |
| CCR7*** | TG8 | eBiosciences |
| CXCR4 | 12G5 | BD Biosciences |
| CLA | HECA-452 | BD Biosciences |
| CCR4 | 1G1 | BD Biosciences |
| ICOS | ISA-3 | eBiosciences |
| ICOS-L | MIH12 | eBiosciences |
| OX40 | ACT35 | BD Biosciences |
| PD-1 | MIH4 | BD Biosciences |
| TCRαβ | WT31 | BD Biosciences |
| TCRγδ | B1 | BD Biosciences |
| TCRγδ | IMMU510 | Thermo Fisher |
| TCRδ1 | TS-1 | Thermo/Pierce |

| | | |
|---|---|---|
| TCRδ2 | B6 | BD Biosciences |
| TCRγ9 | B3 | BD Biosciences |
| invariant NKT | 6B11 | BD Biosciences |
| NMS | 015-000-120 | Jackson ImmunoResearch |
| DNAM1 | DX11 | BD Biosciences |
| NKG2D | 1D11 | BD Biosciences |
| IL15 | 34559 | R&D Systems |
| IFNγ | 4S.B3 | BD Biosciences |
| TNFα | MAb11 | BD Biosciences |

* To detect CAR expression

** To detect CD19-specific CAR expression

*** Used at 1:67 dilution

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**VITA**

Drew Caldwell Deniger was born on January 2, in Dallas, TX to William Southgate Deniger, Jr. and Jeanne Caldwell Deniger. He attended Dallas' Hillcrest High School where he served as the art and design editor for the school's newspaper, *The Hurricane*, and played varsity soccer and football. After graduating from Hillcrest in 1998, he enrolled at The University of Texas at Austin where he earned B.S. degrees in Chemistry and Biochemistry. He then worked as a research associate for three years in the lab of Robert A. Davey, Ph.D. at The University of Texas Medical Branch in Galveston, TX. In 2006, he enrolled at The University of Texas Graduate School of Biomedical Sciences in the M.S. program under the mentorship of Madeleine Duvic, M.D. and graduated two years later with a thesis focused on metastatic melanoma. He then enrolled in the Ph.D. program at The University of Texas Graduate School of Biomedical Sciences and joined the Immunology Program and the laboratory of Laurence J.N. Cooper, M.D., Ph.D. in the Division of Pediatrics at The University of Texas M.D. Anderson Cancer Center.

RAC Review (Dec 4-5 2012)

# A study to infuse ROR1-specific Autologous T cells for patients with CLL

*Presenters*

PIs: Dr. William Wierda and Dr. Laurence JN Cooper, MDACC

Co-PI: Dr. Thomas J. Kipps, UC San Diego

# Targeting ROR1

- **Thomas Kipps MD,PhD**
- **William Wierda MD,PhD**
- **Laurence Cooper MD,PhD**

# ROR1 Is Expressed In CLL, But Not Normal Adult Tissues



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*Blood* (ASH Annual Meeting Abstracts) 2004 104: Abstract 772
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Oral Sessions

## Restricted Expression of the Orphan Tyrosine Kinase Receptor ROR1 in Chronic Lymphocytic Leukemia.

Tetsuya Fukuda, MD, PhD[1,2,*], Desheng Lu, MD, PhD[1,2,*], Dennis A. Carson, MD[1,2] and Thomas J. Kipps, MD, PhD[1,2]

**2004 ASH Annual Meeting. Blood, 2004,104 (11): 772**

## Antisera induced by infusions of autologous Ad-CD154-leukemia B cells identify ROR1 as an oncofetal antigen and receptor for Wnt5a

Tetsuya Fukuda[*†], Liguang Chen[*‡], Tomoyuki Endo[*§], Li Tang[*‡], Desheng Lu[*], Januario E. Castro[*‡], George F. Widhopf II[*‡], Laura Z. Rassenti[*‡], Mark J. Cantwell[*¶], Charles E. Prussak[∥], Dennis A. Carson[*,**], and Thomas J. Kipps[*‡**]

*Moores Cancer Center, and ‡Chronic Lymphocytic Leukemia Research Consortium, University of California at San Diego, La Jolla, CA 92093; and ∥IM Solana Beach, CA 92075

Contributed by Dennis A. Carson, December 28, 2007 (sent for review December 5, 2007)

We examined the sera of six patients before and after i.v. infusions of autologous chronic lymphocytic leukemia (CLL) cells transduced ex vivo with an adenovirus encoding CD154 (Ad-CD154). Five patients made high-titer antibodies against adenovirus and three made IgG reactive with a leukemia-associated surface antigen, which we iden-

**2008, PNAS, 105(8):3047-52**

E7.5 — *Ror1*, *Ror2*
E8.5 — *Ror1* (al), *Ror2* (al)
*Ror1*, *Ror2*
E9.5 — *Ror1*, *Ror2*
E10.5 — *Ror1*, *Ror2*

Matsuda, T. et al, Mech Dev 105-6, 2001
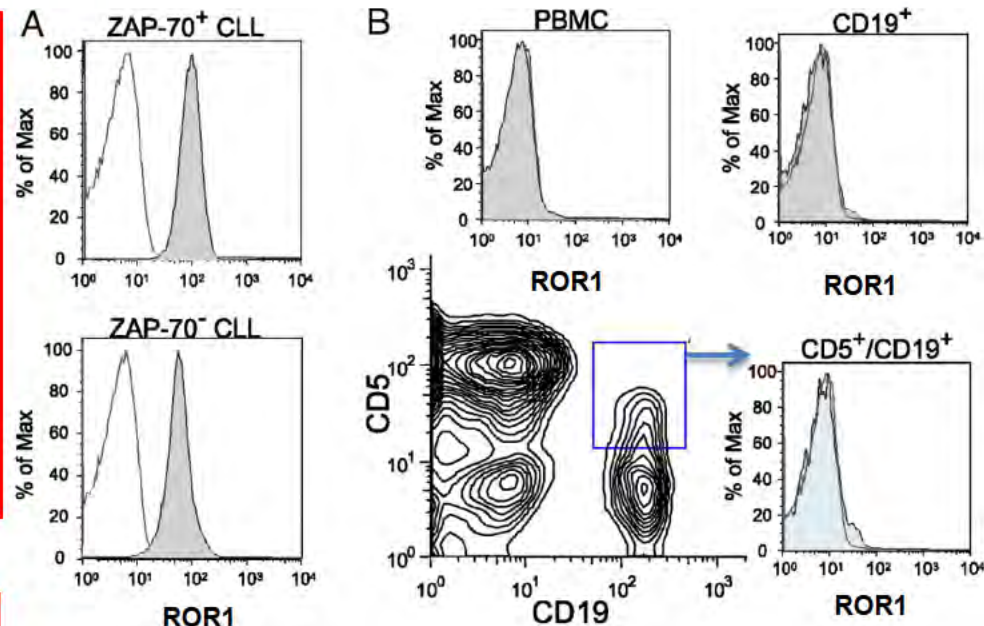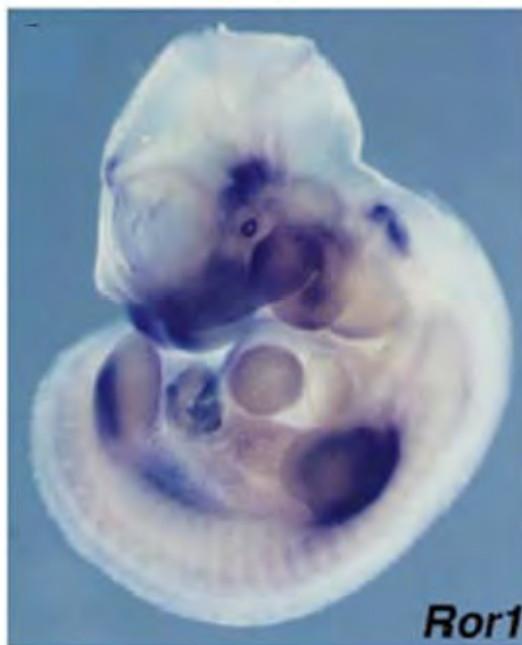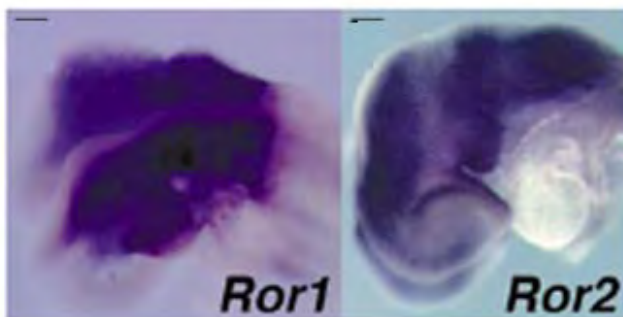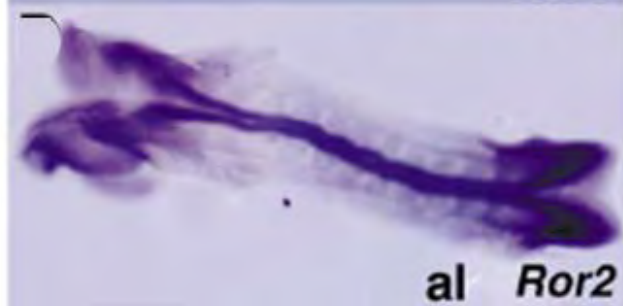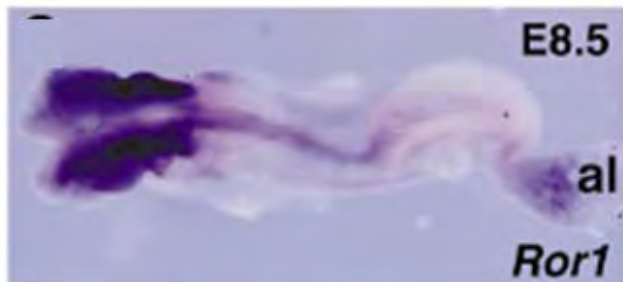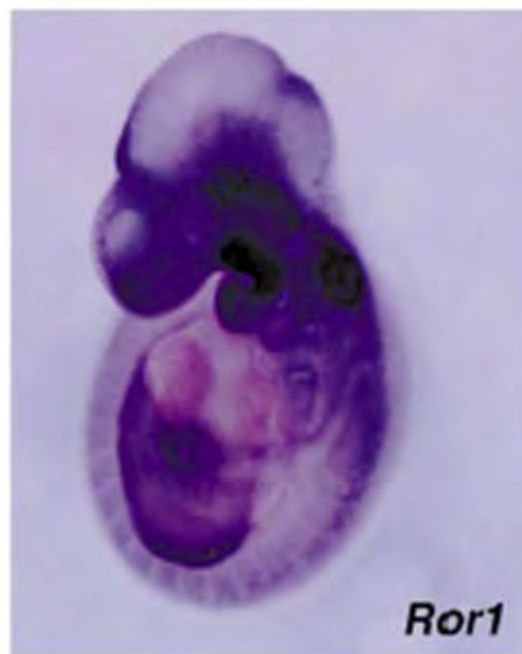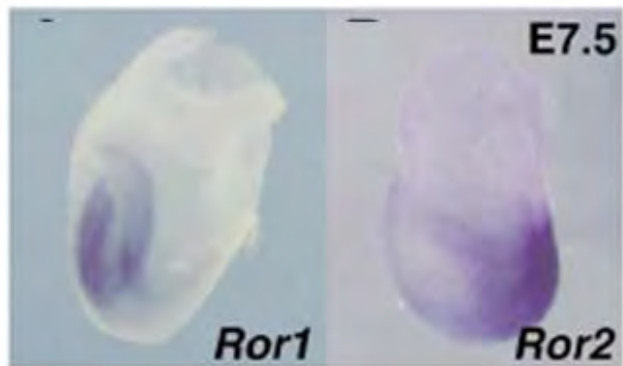
# ROR1 Is Not Expressed In Cord Blood Or Fetal Liver Hematopoietic Stem And Progenitor Cells



Cord Blood Mononuclear Cells
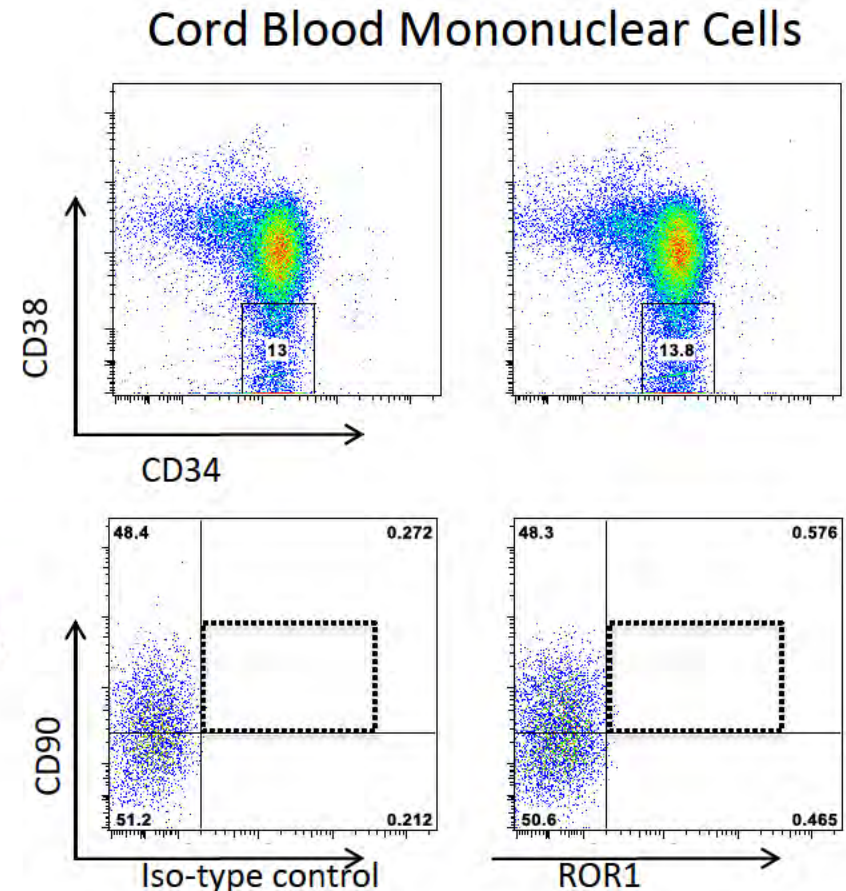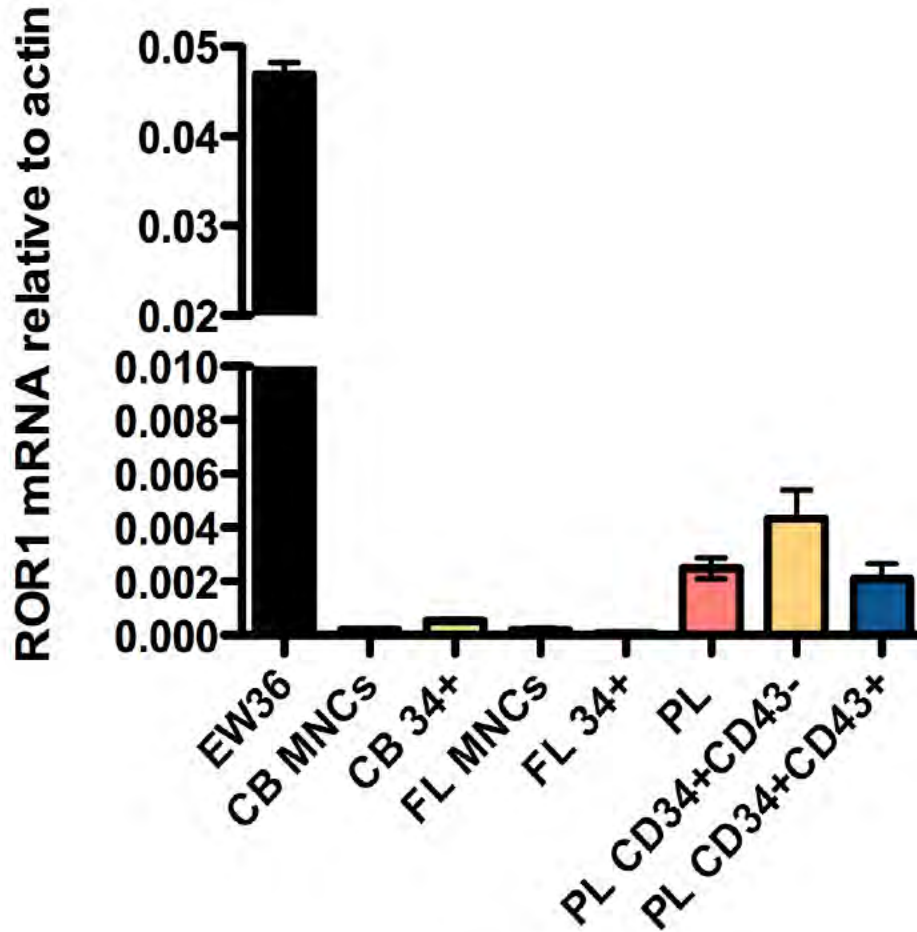
# CD10⁺/CD19⁺/CD34⁻/TdT⁻ Hematogones Express ROR1
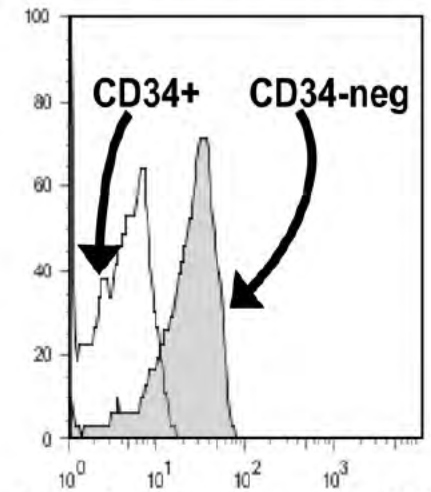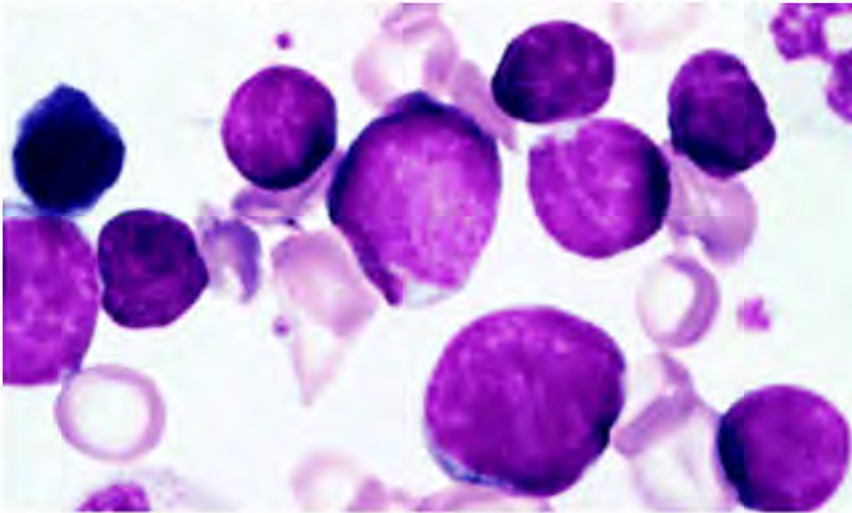


**Blood B-lymphocytes**
(CD5-neg, CD19+)

**CLL**
(CD5+, CD10-neg, CD19+)

**Hematogones**
(CD45-dim, CD10+, CD19+)

CD34+    CD34-neg
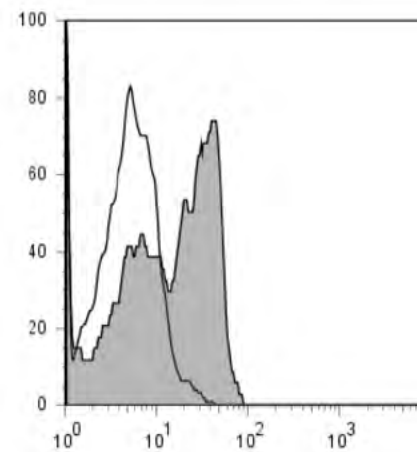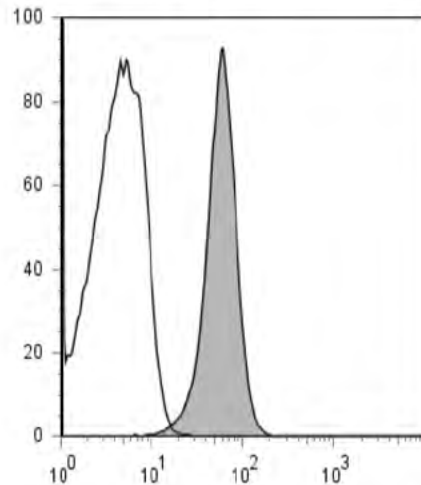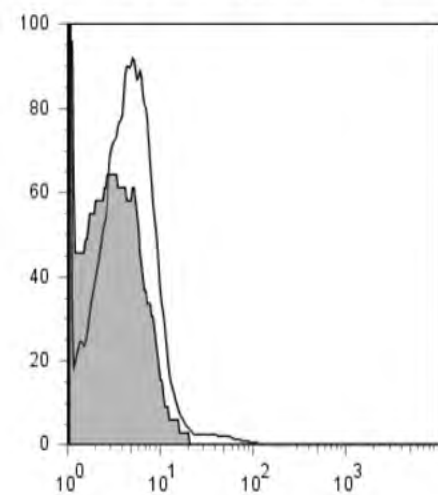
**FL4-H: 4A5-Alexa 647 (ROR1)**

FL4-H: 4A5-Alexa 647 (ROR1)

TdT+    TdT-neg

FL4-H: 4A5-Alexa 647 (ROR1)
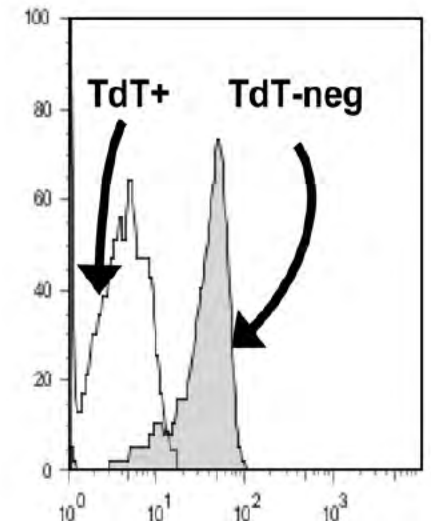
*Broome, E. et al, Leuk Res. 35(10):1390–1394, 2011.*

# Expression Of ROR1 In CLL

The CLL cells of >94% of patients express ROR1 protein, which is not found on normal post-partum tissues

94% of Cases ROR1+ (N=800)



ROR1-   ROR+

MFIR

Significant threshold

CLL samples N=800

4,200 Genes

log2(Expression FPKM)
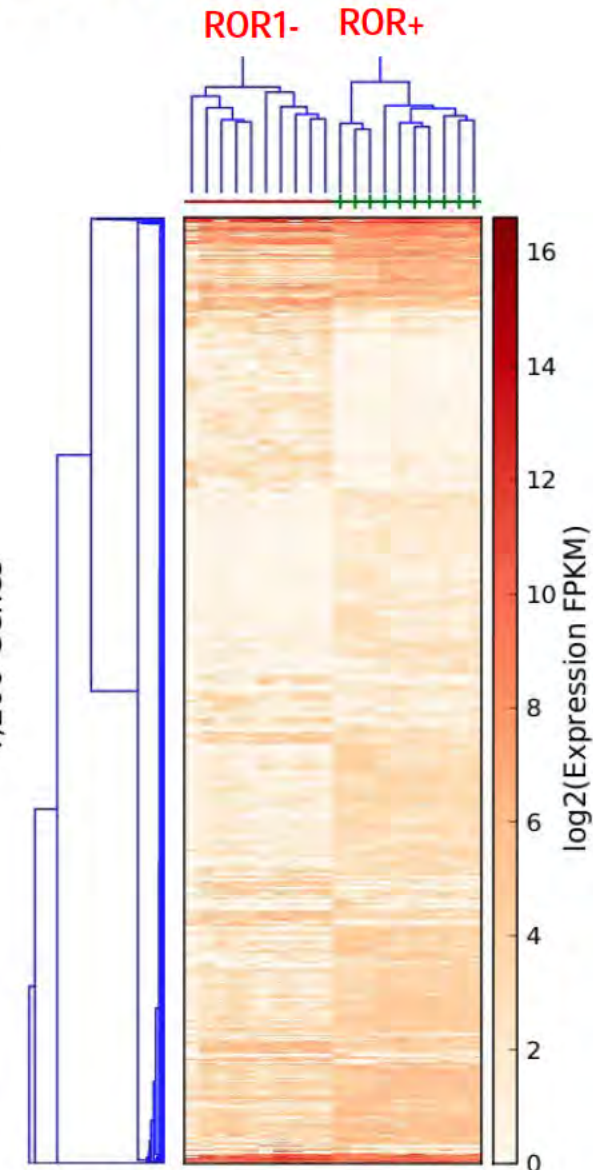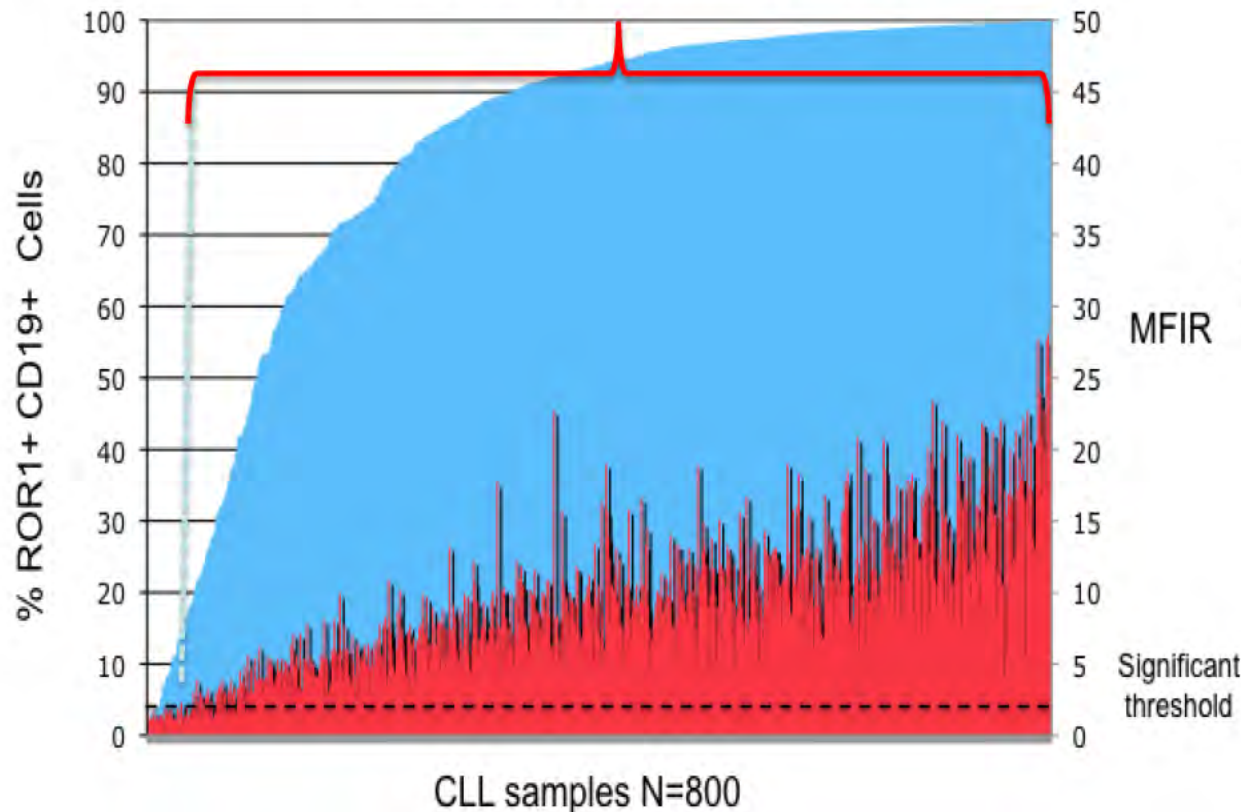
ROR1⁻ CLL has a distinct transcriptome signature

# ROR1 Is A Cancer-Cell Specific Target

- Canadian Genome Center and CIRM Genomics Core performed paired-end RNA sequencing

- ROR1 mRNA was not observed in normal tissues
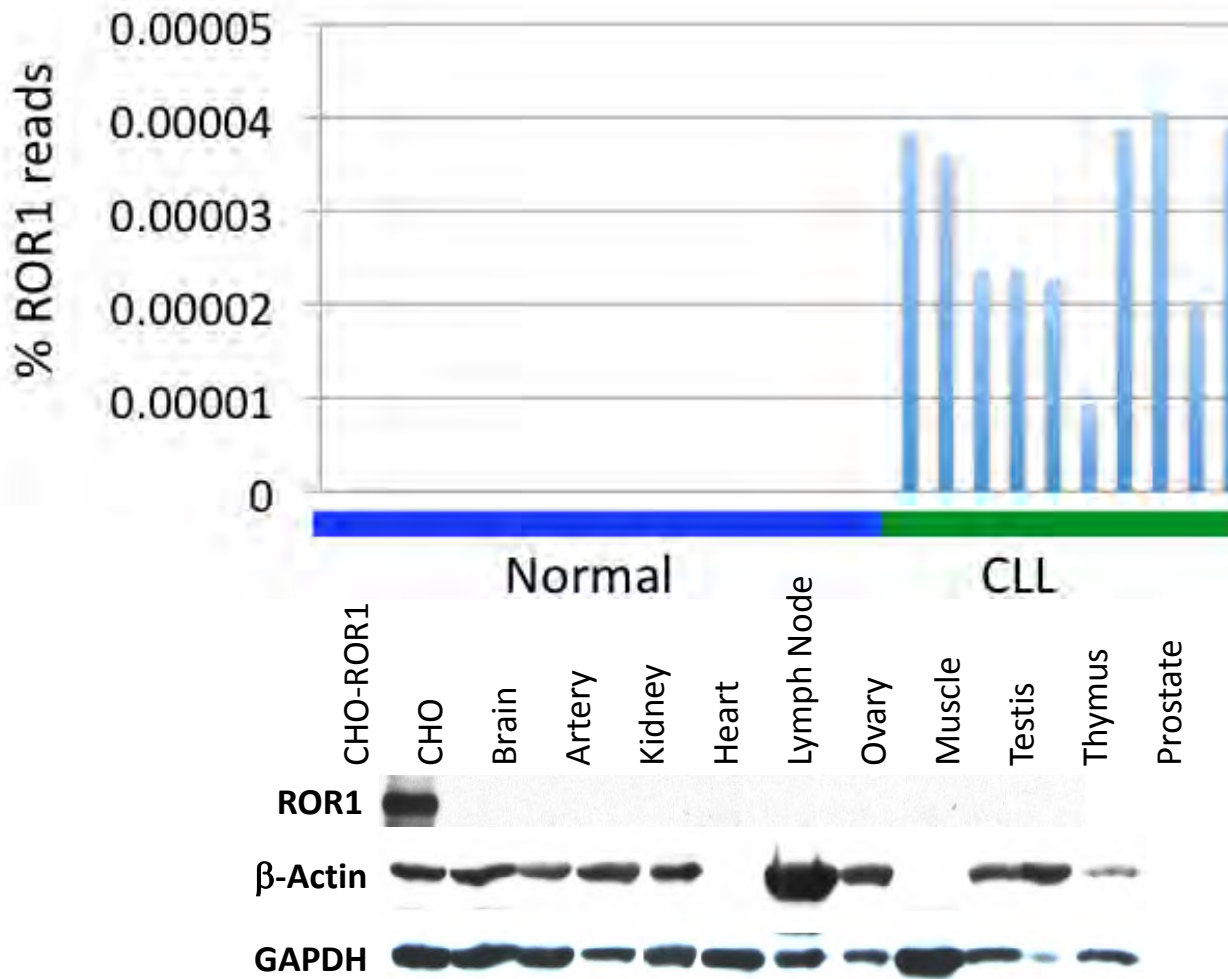
Normal tissues (RNAseq)
- adipose
- adrenal
- white blood cells
- skeletal muscle
- prostate
- lymph node
- lung
- brain
- breast
- colon
- kidney
- heart
- liver
- thyroid
- testes
- ovary

# Specific Anti-ROR1 mAb Does Not Cross React With Normal Adult Tissues
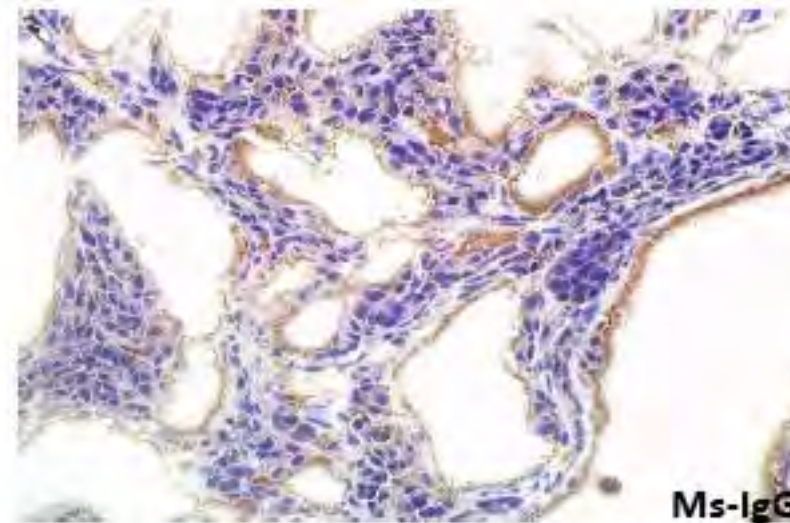
## Normal multi-tissue array (Biomax -FDA999)
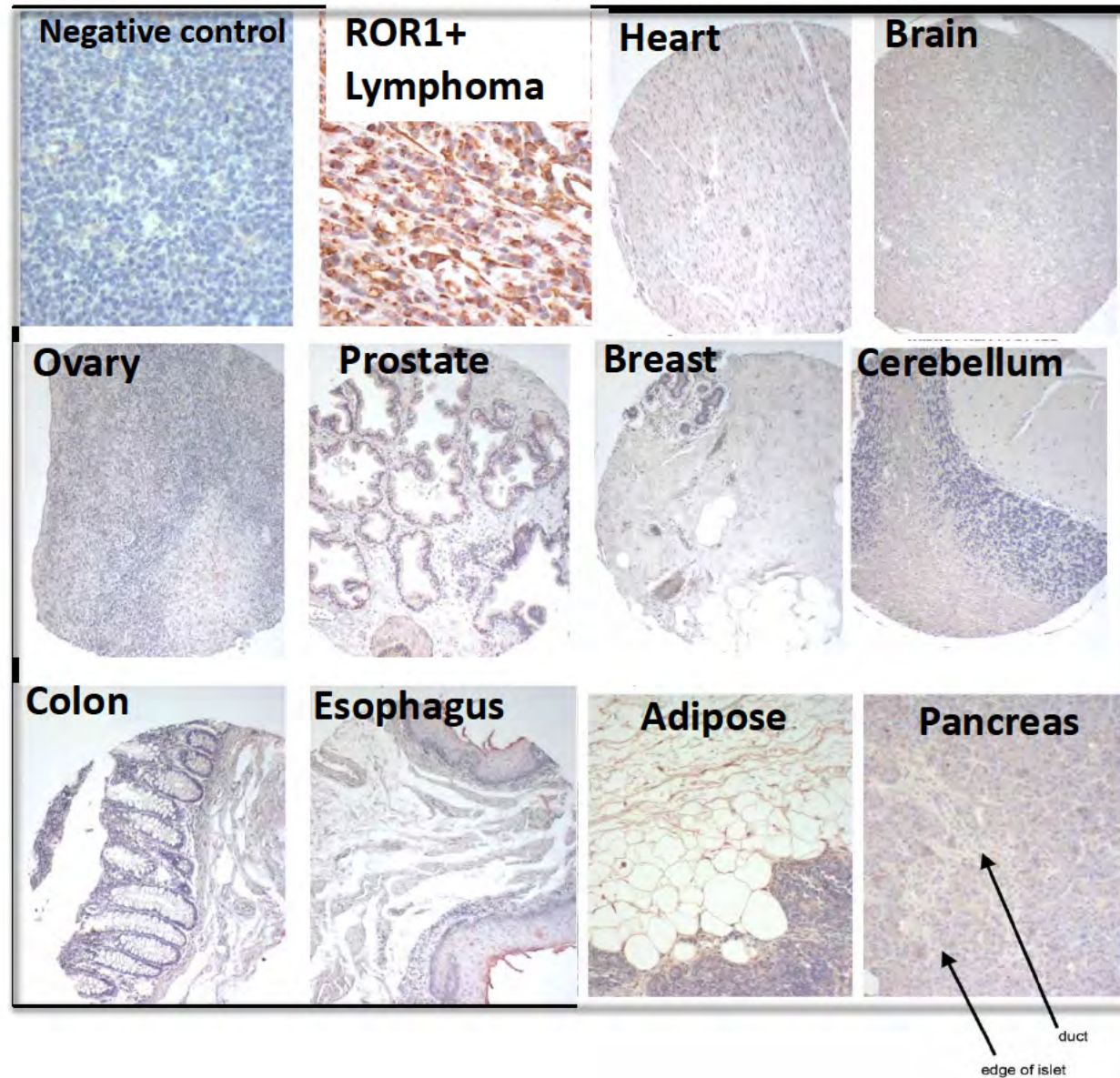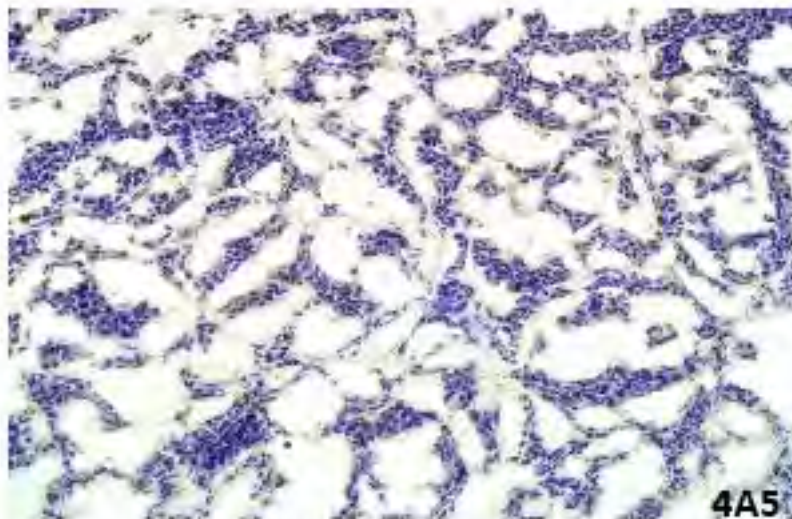
h-Pancreas(D3-0708004) mAb 4A5, 150µg/ml
Frozen tissue, x20, Score: 0
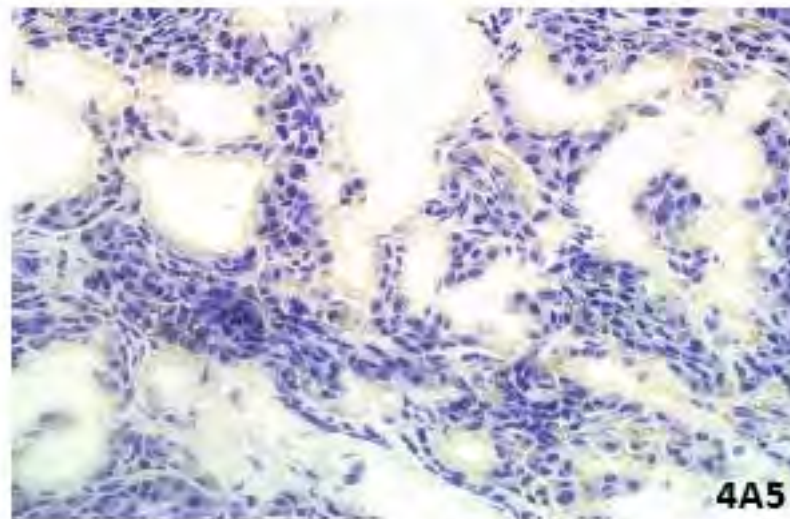
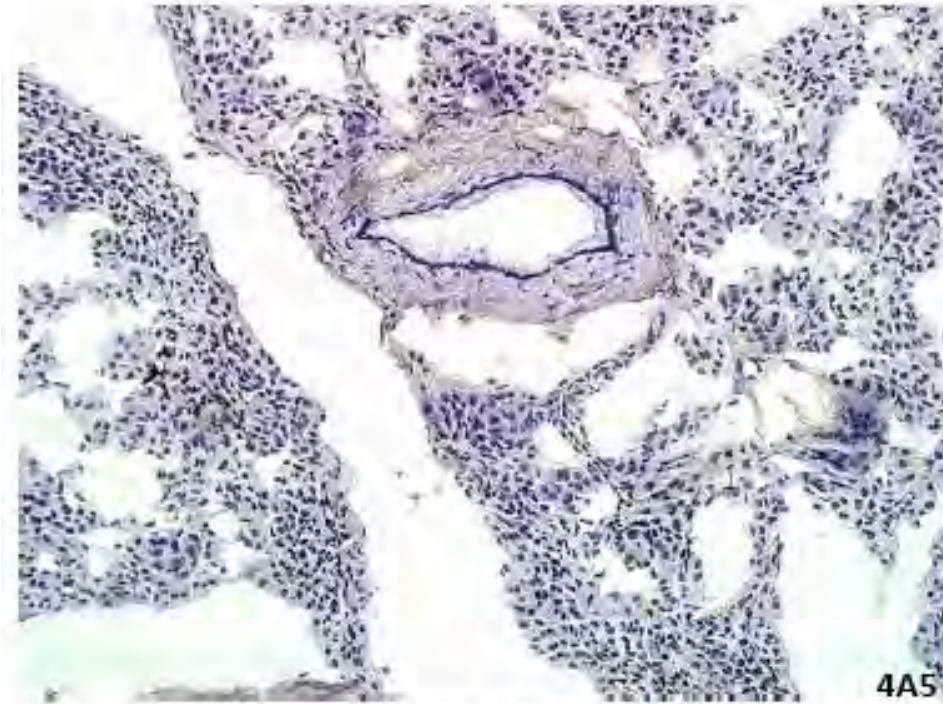h-Pancreas(D3-0708004) mAb 4A5, 150µg/ml
Frozen tissue, x40, Score: 0

h-Pancreas(D3-0708004) Ms-IgG, 150µg/ml
Frozen tissue, x20, Score: 0

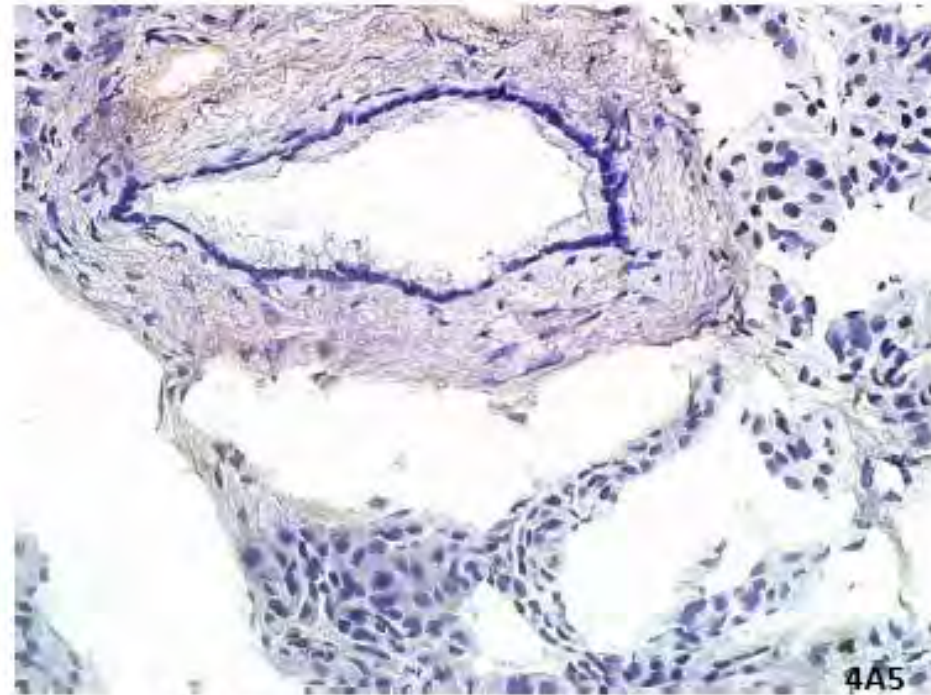h-Pancreas(D3-0708004) Ms-IgG, 150µg/ml
Frozen tissue, x40, Score: 0

*Alireza Ebrahimnejad, PhD, Comparative Biosciences Inc., Sunnyvale, CA 94085*
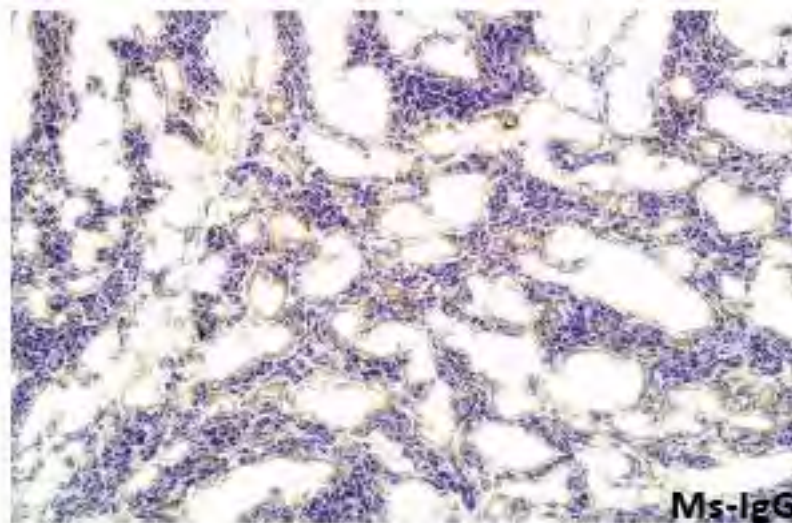
# 4A5 Does Not React With Fresh-Frozen Pancreatic Tissue



h-Pancreas(D3-0708004) mAb 4A5, 150µg/ml
Frozen tissue, x20, Score: 0

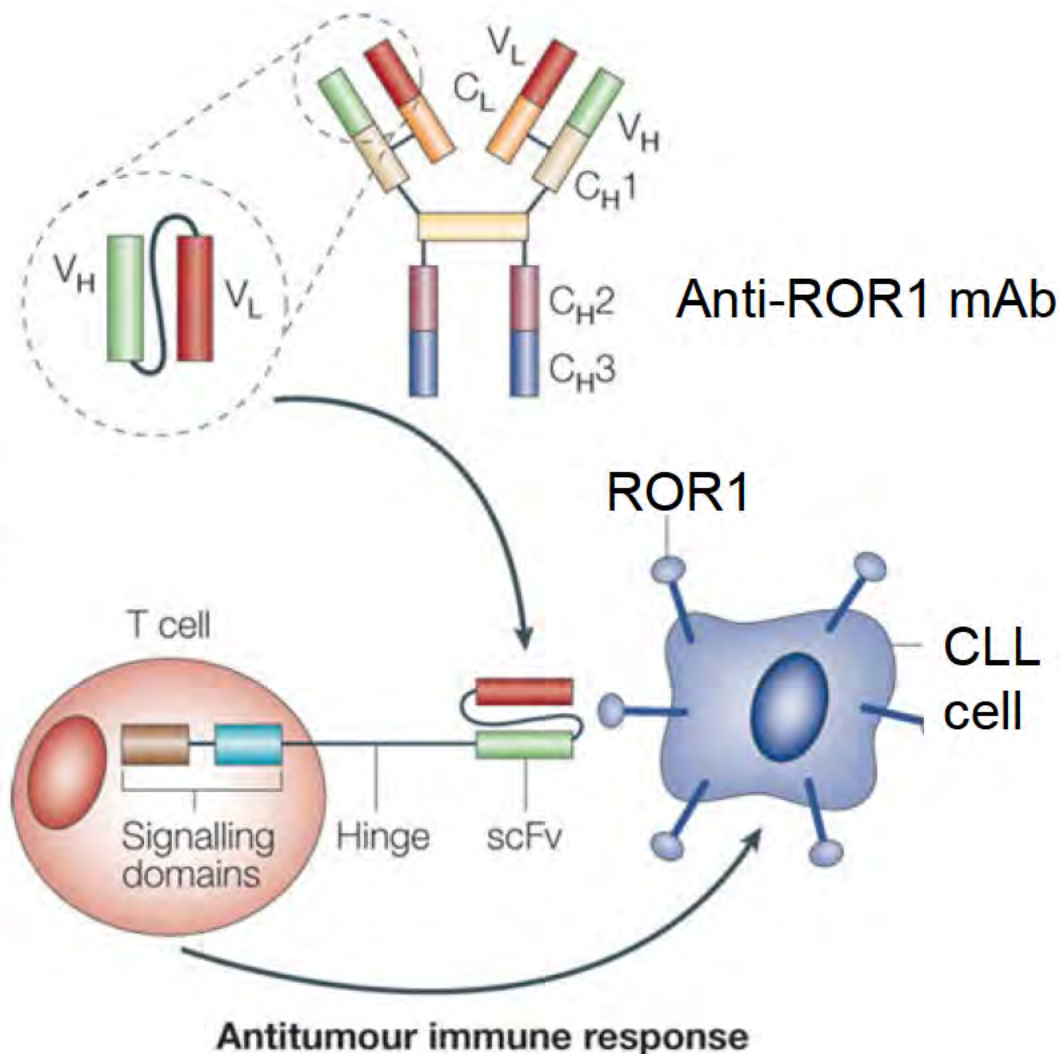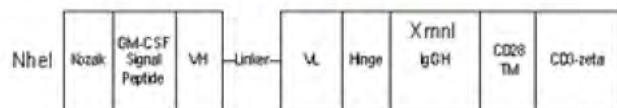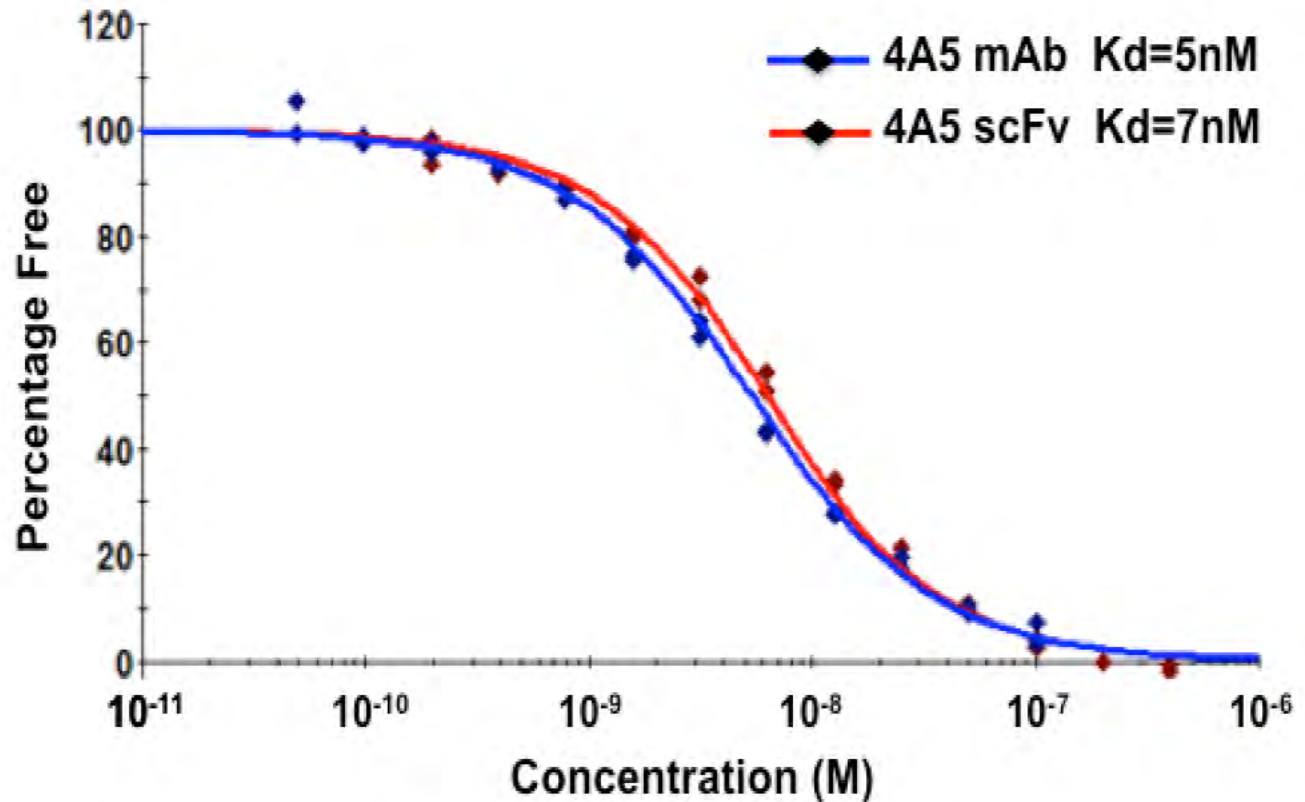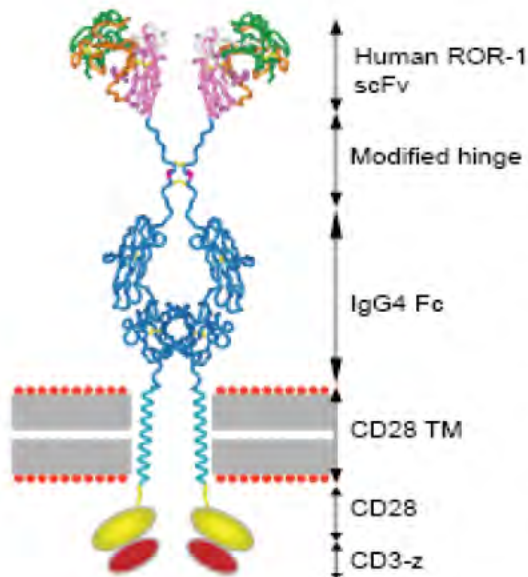h-Pancreas(D3-0708004) mAb 4A5, 150µg/ml
Frozen tissue, x40, Score: 0

*Alireza Ebrahimnejad, PhD, Comparative Biosciences Inc., Sunnyvale, CA 94085*

# Chimeric Anti-ROR1 T-cell Receptors



Anti-ROR1 mAb

ROR1

CLL cell

T cell

Signalling domains

Hinge

scFv

**Antitumour immune response**

CAR Stock

A schematic for the cloning the ROR-1 scFv into the CAR is:

| Nhel | Kozak | GM-CSF Signal Peptide | VH | Linker | VL | Hinge | Xmnl IgGH | CD28 TM | CD3-zeta |

# Affinity Of mAb & Single-chain Variable Region Fragment (scFv) For Recombinant ROR1

# Comparison of Anti-ROR1 mAb and scFv

| | Structure | Reactivity | Kd (10⁻⁹ M) | ROR1 Binding Region |
|---|---|---|---|---|
| 4A5 mAb | Bivalent | Human | 4.5 | Ig Domain |
| 4A5 scFv | Monovalent | Human | 7.4 | Ig Domain |
| 2A2-IgG* | Bivalent | Human/mouse | 0.8 | Ig Domain |
| 2A2-IgG* | Monovalent | Human/mouse | 32.6 | Ig Domain |

*From Sivasubramanian et. al., mAbs 4:3, 1-13 May 2012*

# Summary

- ROR1 is expressed in early embryonic development
- Expression of ROR1 attenuates during fetal development
- Normal post-partum tissues do not express ROR1 except for hematogones, unusual CD5 precursor B cells.
- ROR1 is expressed by virtually all cases of chronic lymphocytic leukemia
- We have generated highly specific, high-affinity mAb specific for human ROR1 from which we generated single-chain anti-ROR1 Fv of high affinity for ROR1
- Unlike anti-ROR1 mAbs or CAR developed by other investigators, our mAb do not cross-react with normal post-partum tissues

# The Clinical Continuum of CLL – Finding Etiology

**Indolent CLL** → **Active CLL** → **Relapsed / Refractory CLL** → **Richter's Transformation**

"Early" asymptomatic

"Later" symptomatic

"Late" active, resistant, limited disease control

Progressive, refractory disease

Healthy ▶ MBL

Healthy at risk

# Outcomes in
# Relapsed / Refractory CLL

# Responses to Regimens for Relapsed or Refractory CLL

| Regimen | n | % Pts | % CR | % OR |
|---|---|---|---|---|
| CIT | 244 | 49 | 23 | 67 |
| mAb | 101 | 20 | 11 | 42 |
| Lenalidomide | 32 | 6 | 6 | 34 |
| Len + CD20 mAb | 73 | 15 | 4 | 62 |
| Other | 51 | 10 | 0 | 22 |
| Overall | 501 | | 14 | 54 |

# Response to Salvage Treatment* by FISH

| FISH | n | % Pts | % CR | | % OR | |
|------|---|-------|------|---|------|---|
| 17p del | 106 | 28 | 7 | p=.013 | 40 | p<.001 |
| 11q del | 97 | 26 | 11 | | 63 | |
| +12 | 44 | 12 | 20 | | 64 | |
| None | 61 | 16 | 26 | p=.002 | 56 | |
| 13q del | 66 | 18 | 12 | | 68 | p=.04 |
| Overall | 374 | | 14 | | 56 | |

*Includes: CIT; mAb; revlimid; revlimid+mAb; other

# Multivariable Model for Time-to-Treatment Failure: Salvage Treatment (n=299)

| Characteristic | Relative Risk | p-value |
|---|---|---|
| 17p del *vs.* Others | 1.58 | .007 |
| # Prior CLL Treatments | 1.22 | <.0001 |
| *IGHV* Status (UM *vs.* Mutated) | 1.94 | .0009 |
| HGB (gm/dl) | .86 | .002 |
| Chemoimmunotherapy *vs.* Others | .47 | <.0001 |
| Lenalidomide +CD20 mAb *vs.* Others | .32 | <.0001 |

# TTF by Treatment: Salvage (N=501)

| Salvage Treatment | Events | Censored |
|---|---|---|
| Chemoimmunothearapy | 115 | 129 |
| Monoclonal Ab | 77 | 24 |
| Other | 29 | 22 |
| Revlimid | 31 | 1 |
| Revlimid + CD20 mAb | 27 | 46 |

Proportion of Patients

Months
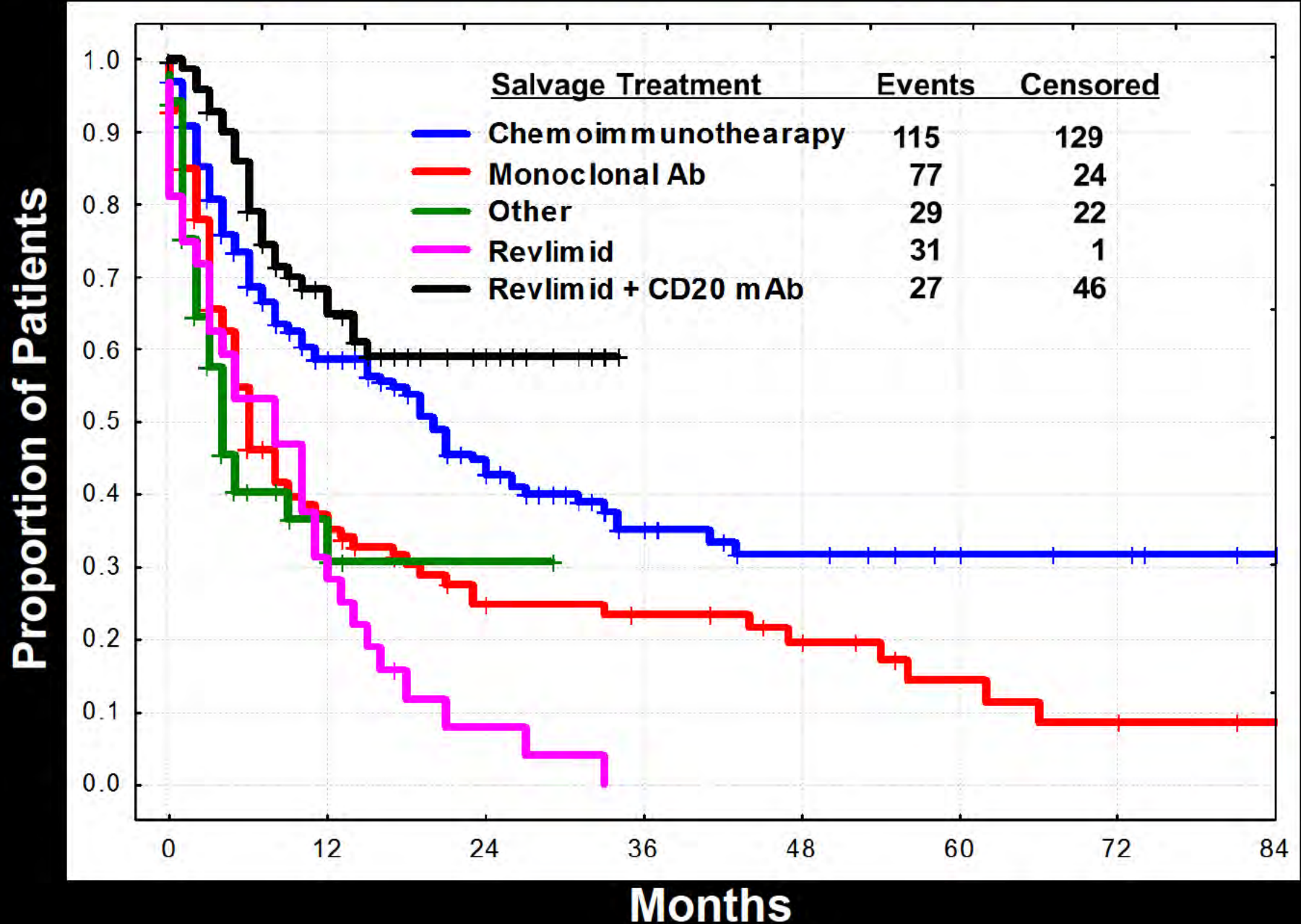
# Goals of the proposed trial

1. ## Avoid targeting of CD19 on normal B cells
   - One patient death due to opportunistic viral infection at NCI in patient that received CD19-specific CAR$^+$ T cells
     - *Kochenderfer et al., Blood. 2012 Mar 22;119(12):2709-20; Cooper et al., Blood. 2012.*

2. ## Determine the CAR design that improves therapeutic potential
   - It is not known whether signaling through CD28/CD3-zeta or CD137/CD3-zeta is superior and this can only be tested in human trials
     - Both CAR designs target normal CD19$^+$ B cells and have exhibited anti-tumor effects
     - Therapeutic potential is correlated with persistence

# Precedent for competitive repopulation experiment co-infusing two populations of CAR⁺ T cells

- Patients with non-Hodgkin' lymphoma (NHL) were simultaneously infused with 2 autologous T-cell products (CAR-CD19ζ and CAR-CD19-28 ζ)
- Objective was to determine the *in vivo* effects of co-stimulatory endodomain on fate of CAR-engineered T cells
- CD28 co-stimulation improves the *in vivo* expansion and persistence of CAR-modified T cells

Savoldo B, et al. J Clin Invest. 2011 May;121(5):1822-6.

# Phase I ROR1-CAR-T for Rel/Ref CLL

- ## Rationale:
  - Surface ROR1 ubiquitously expressed on CLL B cells, not on normal B cells or normal tissues
  - CD19-CAR-T cells have clinical activity and were tolerated
  - Limited long-term disease control options for relapsed CLL

- ## Previously treated CLL/SLL:
  - Adequate kidney and liver fctn.; no active infection or autoimmunity; adequate counts; no pregnancy

- ## Primary endpoint:
  - 3+3 dose escalation to MTD
  - ROR1-CAR-T with 3 dose levels
  - Expansion to 12 pts
  - Multi-center study – MDACC & UCSD

- ## Secondary endpoints:
  - Tolerability, toxicities, response rate (CR/PR/OR), TTF, TTP, OS
  - Correlative laboratory studies

# Phase I ROR1-CAR-T for Rel/Ref CLL

**Primary Objective:**

- Describe safety profile by identifying MTD and record *in vivo* persistence of autologous *ex vivo* expanded ROR1-specific T cells

**Secondary Objectives:**

- Assess feasibility of infusing autologous ROR1-specific T cells
- Obtain indication of clinical therapeutic activity by overall response (OR) rate [complete response (CR) & partial response (PR) rates]
- Study persistence of infused autologous ROR1-specific T cells
- Screen for development of host immune responses against the ROR1-specific CAR
- Additional correlative studies:
  - (i) trafficking of genetically modified T cells
  - (ii) development of oligoclonal sub-population(s) of infused T cells
  - (iii) emergence of genetically modified T cells with central memory, stem-cell, and/or naïve immunophenotypes
  - (iv) maintenance of ROR1R-redirected effector functions

# Phase I ROR1-CAR-T for Rel/Ref CLL

**Inclusion Criteria:**

1. Patients with B cell CLL/SLL who have a 2008 NCI-WG/IWCLL indication for treatment

2. Patients who have been previously treated with a standard regimen or untreated with 17p del by FISH (high-risk)

3. At least 21 days from last cytotoxic chemotherapy

4. ECOG PS <2

5. Adequate hepatic function, defined as SGPT <3 x upper limit of normal (ULN); serum bilirubin and alkaline phosphatase <2 x ULN, or considered not clinically significant by the study doctor or designee.  Lower than 2 x ULN is deemed as not clinically significant by the study doctor.

6. Able to provide written informed consent

# Phase I ROR1-CAR-T for Rel/Ref CLL

**Exclusion Criteria:**

1. Positive βHCG in female of child-bearing potential (not post-menopausal for 12 months or no previous surgical sterilization or lactating females)

2. Patients with known allergy to bovine or murine products

3. Positive serology for HIV

4. Active, uncontrolled autoimmune phenomenon (AIHA, ITP) requiring steroid therapy

5. Presence of ≥G3 toxicity (CTC v4) from the previous Rx

6. Concomitant investigational agents

7. Prior allogeneic hematopoietic stem-cell transplantation if evidence of donor chimerism persists (patients with exclusively autologous hematopoiesis will be eligible)

8. Refusal to participate in the long-term follow-up protocol (2006-0676)

# Phase I ROR1-CAR-T for Rel/Ref CLL

- **Treatment (MDACC & UCSD):**
  - **Collect autologous T cells for transduction and expansion (blood or pheresis)**
  - **Lymphodepletive condition with standard-dose FCR, FRB, or BR (D-5 to D-3)**
  - **Split-dose (D0=1/4 then D1=3/4) infusion of ROR1-CAR-T cells**
    - **$1\times10^5$ transduced ROR1-CAR-T cells/kg (50% ROR1/CD28/CD3$\zeta$ & 50% ROR1/CD137/CD3$\zeta$)**
    - **$1\times10^6$ transduced ROR1-CAR-T cells/kg (50% ROR1/CD28/CD3$\zeta$ & 50% ROR1/CD137/CD3$\zeta$)**
    - **$1\times10^7$ transduced ROR1-CAR-T cells/kg (50% ROR1/CD28/CD3$\zeta$ & 50% ROR1/CD137/CD3$\zeta$)**

# Phase I ROR1-CAR-T for Rel/Ref CLL Lymphodepletive Chemoimmunotherapy

| Day of Rx | Fludarabine, Cyclophosphamide, Rituximab (FCR) Regimen | Fludarabine, Bendamustine, Rituximab (FBR) Regimen | Bendamustin, Rituximab (BR) Regimen |
|---|---|---|---|
| D -5 | • Fludarabine 25mg/m$^2$<br>• Cyclophosphamide 250mg/m$^2$<br>• Rituximab 375-500mg/m$^2$ | • Fludarabine 20mg/m$^2$<br>• Bendamustine 30-50mg/m$^2$<br>• Rituximab 375-500mg/m$^2$ | |
| D -4 | • Fludarabine 25mg/m$^2$<br>• Cyclophosphamide 250mg/m$^2$ | • Fludarabine 20mg/m$^2$<br>• Bendamustine 30-50mg/m$^2$ | • Bendamustine 70-90mg/m$^2$<br>• Rituximab 375-500mg/m$^2$ |
| D -3 | • Fludarabine 25mg/m$^2$<br>• Cyclophosphamide 250mg/m$^2$ | • Fludarabine 20mg/m$^2$<br>• Bendamustinem30-50mg/m$^2$ | • Bendamustine 70-90mg/m$^2$ |
| D -2 | | | |
| D -1 | | | |
| D 0 | T cell infusion (25% of the genetically modified cells) | | |
| D +1 | T cell infusion [remainder (75%) of ROR1R-CAR-T cell dose] | | |

# Phase I ROR1-CAR-T for Rel/Ref CLL

| Evaluation | Within 30 (±7) days of T-cell Collection | Within 14 (±7) days of T-cell Infusion | Within 24 hours post 1st & 2nd part of split T-cell infusion (for Day 0 and Day 1) | Within 1-3 days post T-cell infusion | At 2 weeks (±3 days) | At 3 weeks (±3 days) | At 1 month (±5 days) | At 3,6,12 months (±14 days) |
|---|---|---|---|---|---|---|---|---|
| History and physical exam, including weight and vital signs (BP, HR, Temp) | X | X | X | X | X | X | X | X |
| Laboratory examinations: CBC, diff, PLT, ALC, T bili, SGPT or SGOT, Alk Phos, LDH, Alb, total protein, BUN, creatinine, $NA^+$, $CL^-$, $K^+$, $CO_2$, glucose | X | | X | X | X | X | X | X |
| PT/PTT | X | X | | | | | | |
| Serum immunoglobulin | X | X | | | X | | X | X |
| Baseline: chest x-ray, 12-lead ECG | X | | | | | | | |
| Serum pregnancy test, if applicable | X | X | | | | | | |
| Serology for CMV, HIV, HTLV, I/II, Hep B, Hep C, West Nile Virus, RPR, and T. cruzei | X | | | | | | | |
| Disease status to include CT and/or PET scan, bone marrow biopsy, molecular tests for MRD as clinically indicated | | X | | | | | | X |
| Serum for HAMA | | X | | | | | | |
| HLA typing | | X | | | | | | |
| Blood flow cytometry for T- & B-cell markers | | X | | | | | | |
| Adverse events assessment & grading | X | X | X | X | X | X | X | X |
| Research Tests | | X | X | X | X | X | X | X |

# Safety

- Inter-patient dose escalation
- Splitting the infusion of T cells over two days
- "Pause" between patients
- Plan to provide corticosteroids and other immunosuppressive agents

# CAR⁺ T-cell trials at MDACC

| Trial | Agent | NIH-OBA | IND | Enrollment |
|---|---|---|---|---|
| ALL and lymphoma | Autologous CD19-specific T cells | 0804-922 | 14193 | 4 |
| ALL and lymphoma | Allogeneic CD19-specific T cells | 0910-1003 | 14577 | 6 |
| ALL and lymphoma | Allogeneic CD19-specific UCB-derived T cells | 1001-1022 | 14739 | Pending activation |
| CLL | Autologous CD19-specific T cells | 1201-1142 | 15180 | Pending activation |
| CLL | Autologous ROR1-specific T cells | 1210-1192 | Pending | Pending approval |

**2nd generation CD19-specific CAR signaling through CD28 and CD3-ζ**

# MDACC approach to generating clinical-grade CAR⁺ T cells
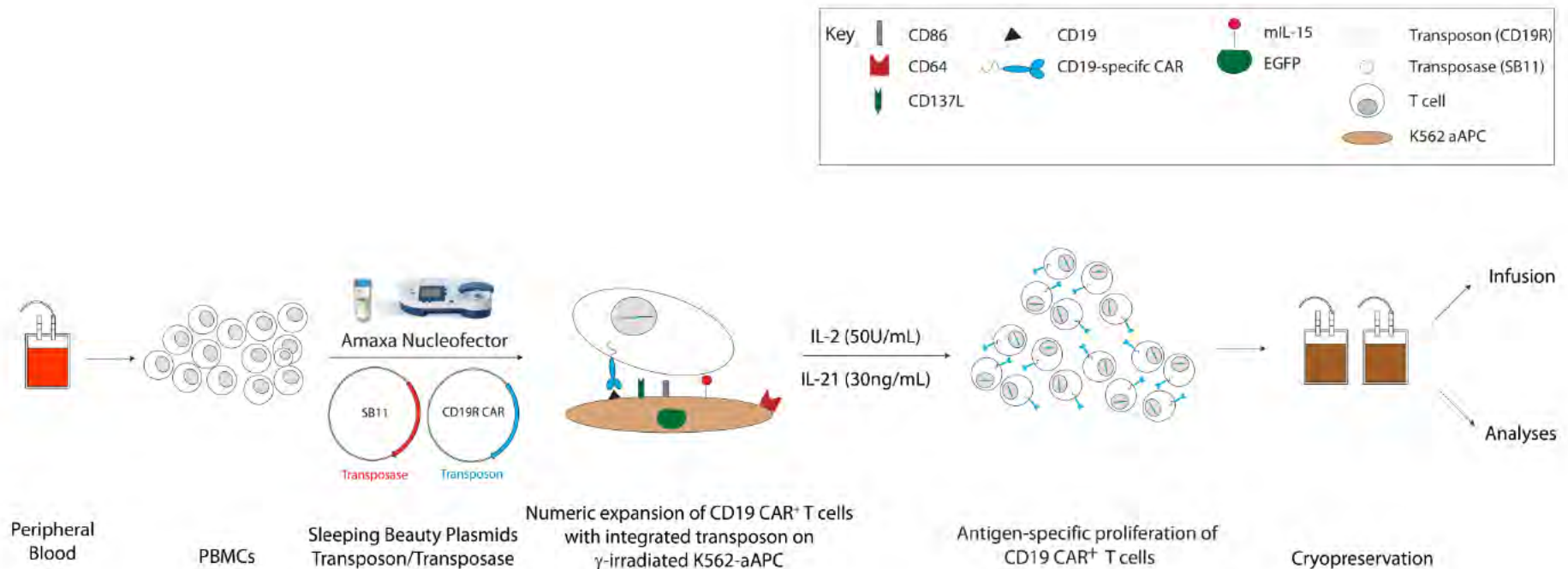
# Sleeping Beauty (SB) Transposition :
# An alternative to retroviral based gene therapy
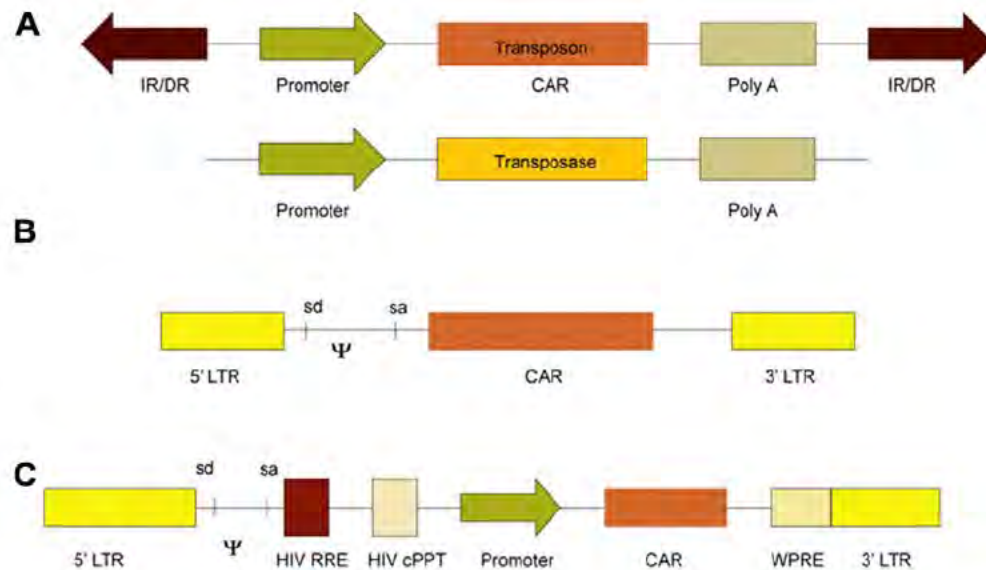


Fig 1. Vectors employed in CAR based gene therapy trials
*Jena B, Dotti G, Cooper L et al., Blood. 2010*
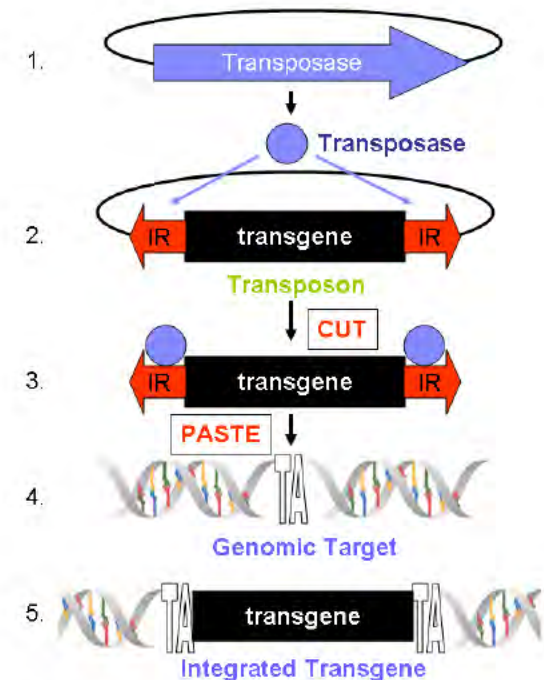
Fig 2. *Sleeping Beauty* transposition
*Switzer K, Rabinovi ch B and Cooper et al., In Press*

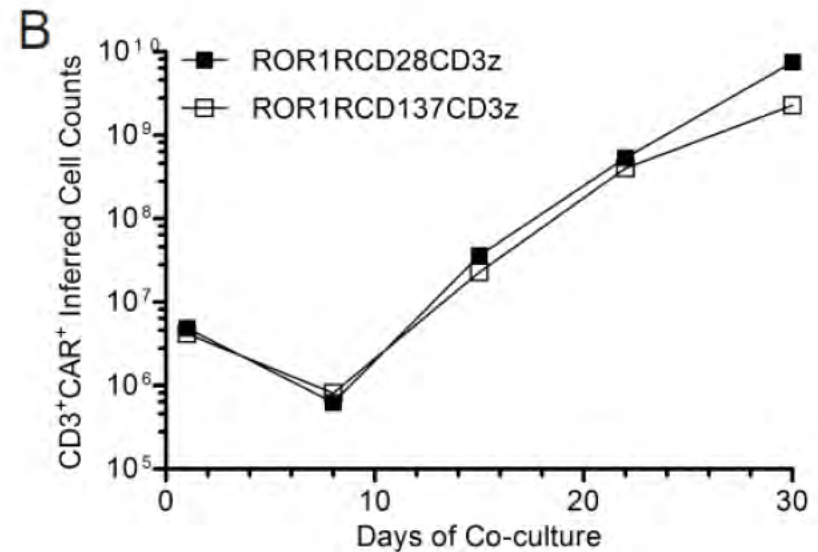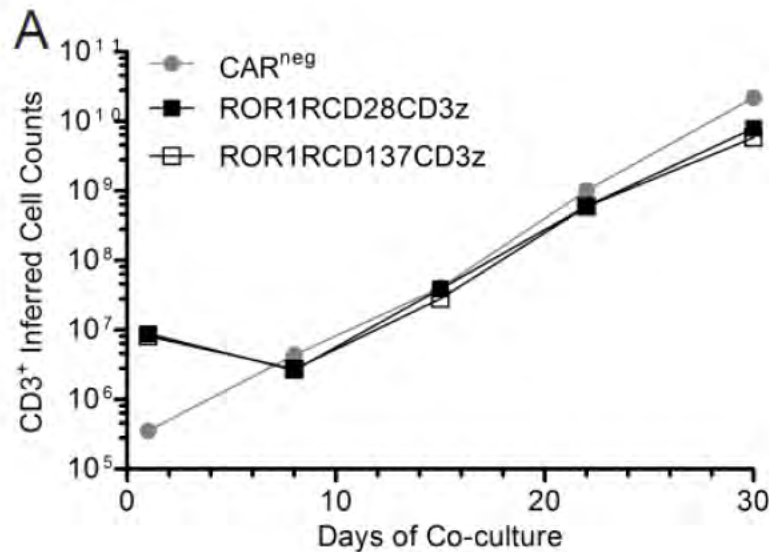# ROR1+ K562-aAPC (Clone 1) Expression of transgene
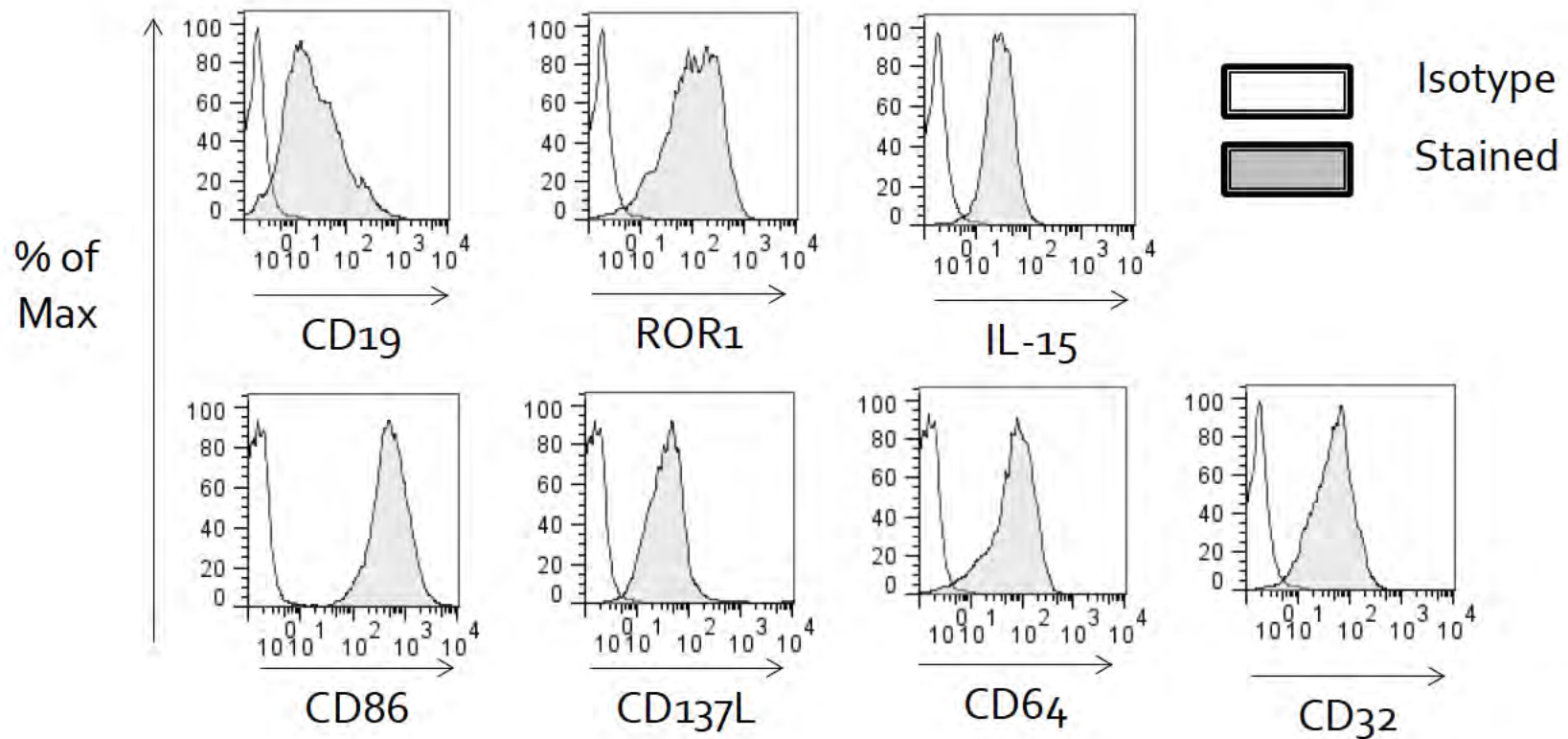
# Co-infusion of two CARs
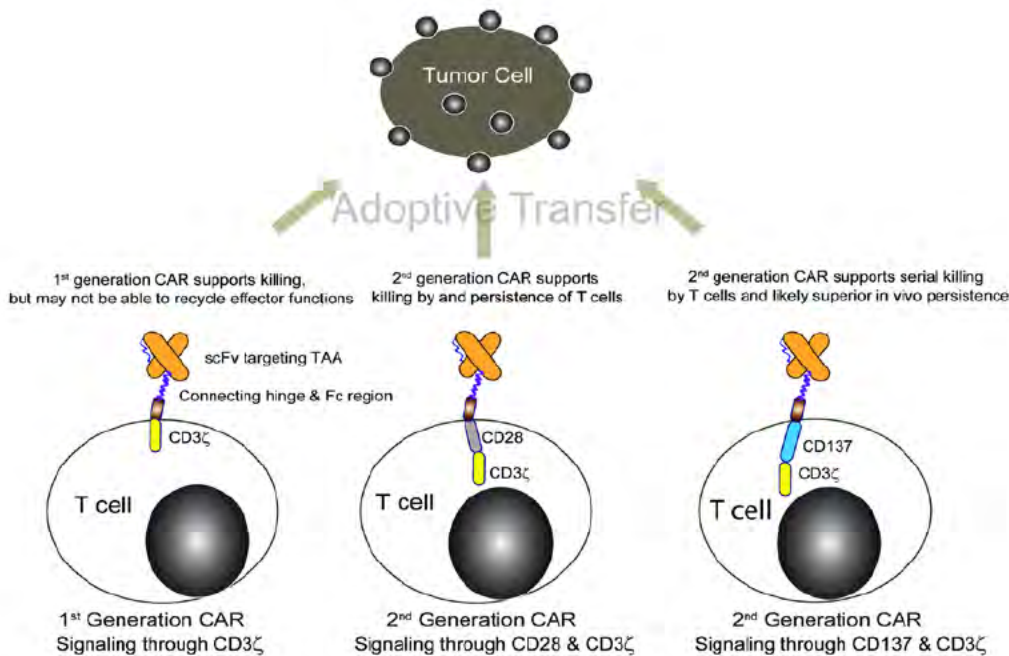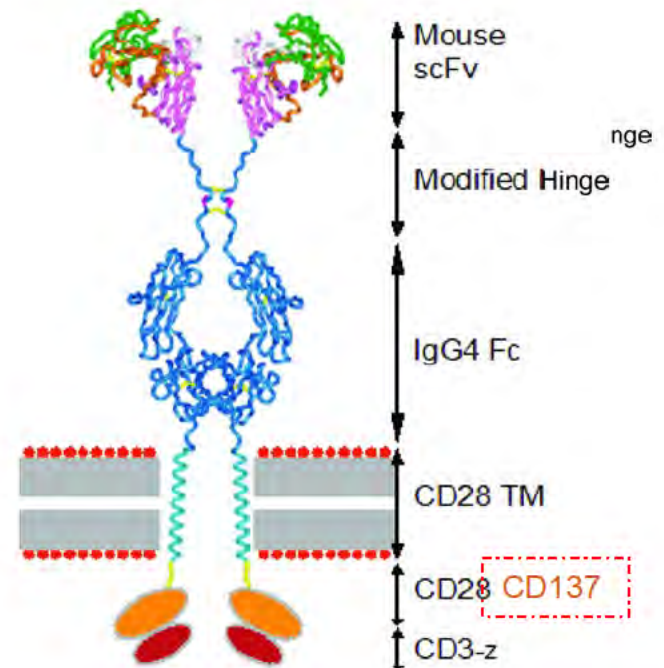
# *Sleeping Beauty* (SB) plasmids
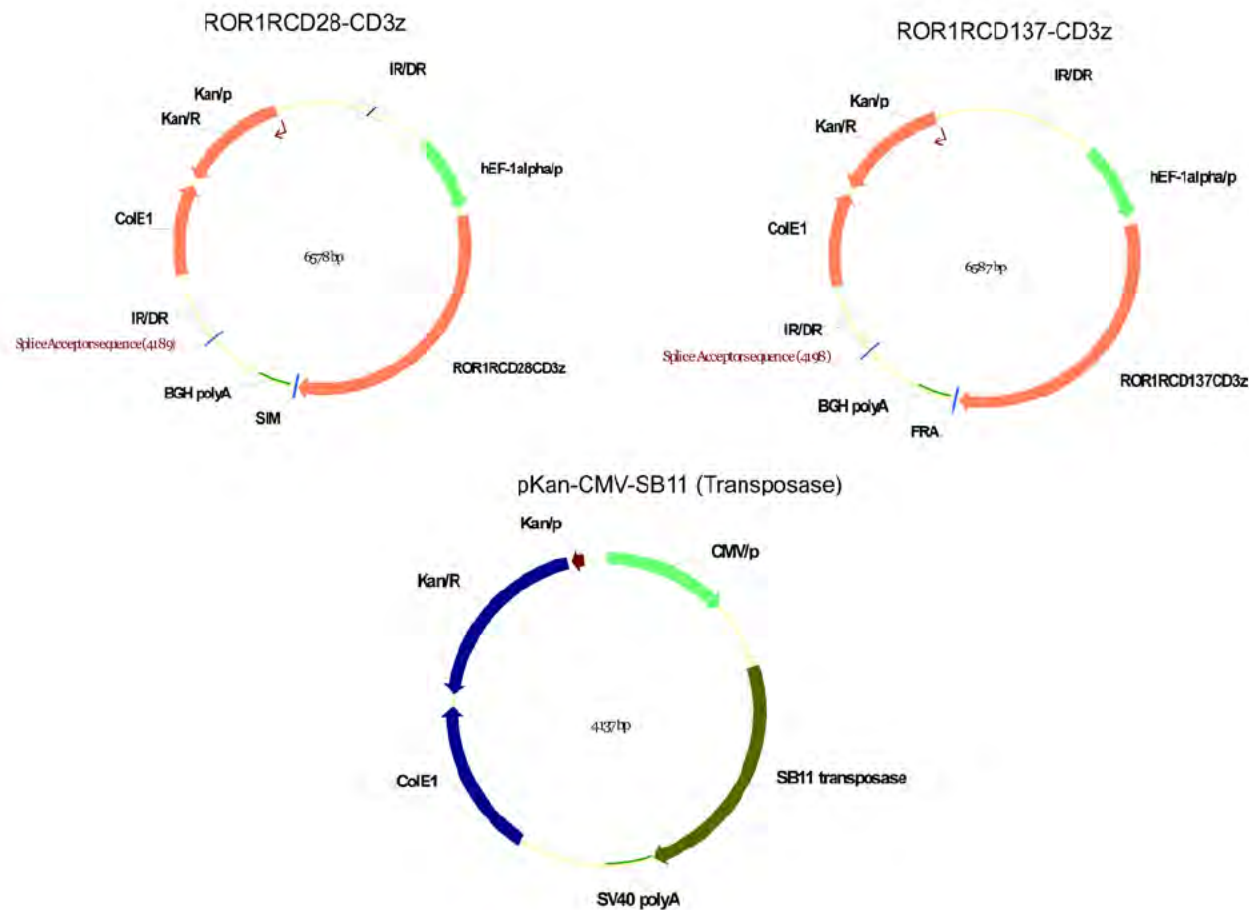
# Propagation of ROR1RCD28 and ROR1RCD137 modified CAR⁺ T cells on aAPC

# Emergence of $\gamma\delta$-T cells in ROR1CD137 CAR$^+$ T cells
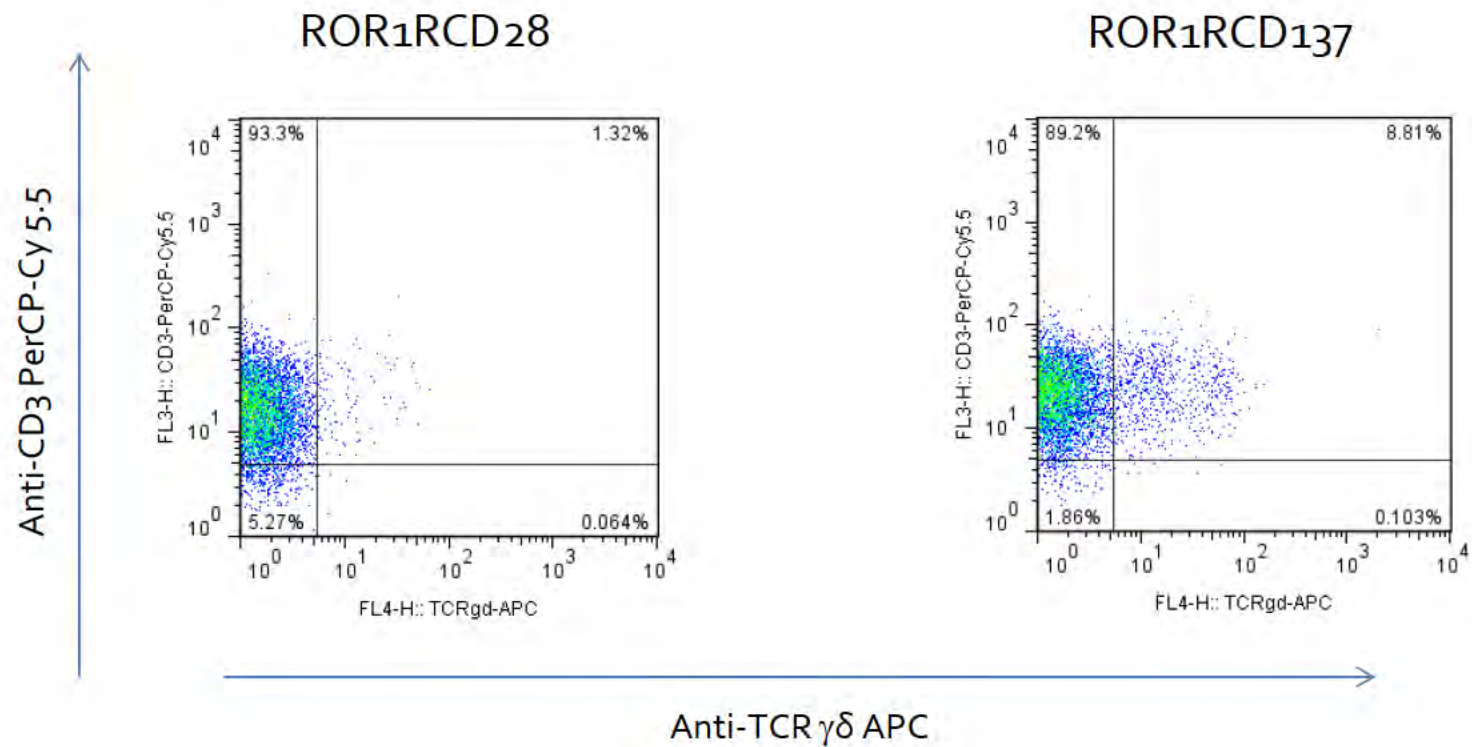
# Redirecetd lysis by ROR1-specific CAR⁺ T cells

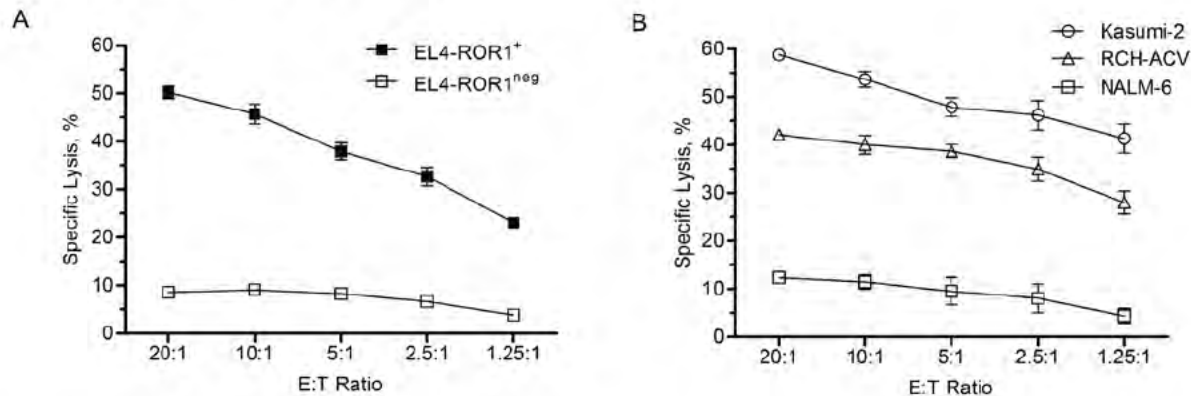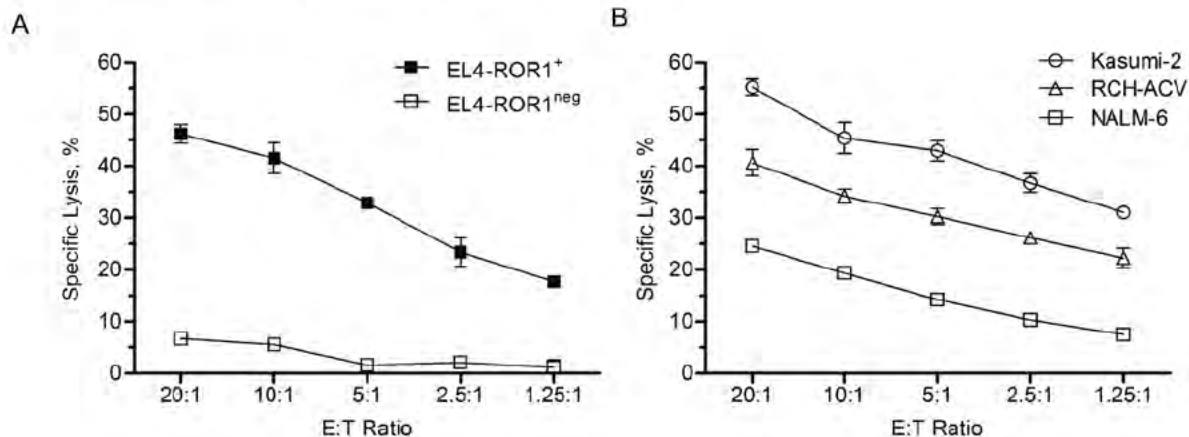Figure 1    Cytotoxic specificity of ROR1RCD28-T cells



Figure 2    Cytotoxic specificity of ROR1RCD137-T cells

# Summary

- Protocol addresses important questions:
  - Avoids inadvertent targeting of normal B cells
  - Tests CAR designs that imparts improved T-cell persistence and thus therapeutic potential
  - Provides investigational immunotherapy for patients with aggressive CLL
- Builds upon our current portfolio of CAR$^+$ T cells
  - Iterative changes to the electroporation and propagation of CAR$^+$ T cells

# Acknowledgements

- MDACC
- UC San Diego
- U MN
- U PENN

# Thanks